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TRACING AMINO ACID METABOLISM OF HARBOR SEALS (*PHOCA VITULINA*) USING STABLE ISOTOPE TECHNIQUES

A

DISSERTATION

Presented to the Faculty

of the University of Alaska

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Liying Zhao, B.A., M.S.

Fairbanks, Alaska

August 2002

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TRACING AMINO ACID METABOLISM OF HARBOR SEALS (*PHOCA VITULINA*)

USING STABLE ISOTOPE TECHNIQUES

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Liying Zhao

RECOMMENDED:

Advisory Committee Chair

Program Head

APPROVED:

Dean, School of Fisheries and Ocean Sciences

Dean of the Graduate School

8-14-02

Date

ABSTRACT

Compound specific isotope techniques were used to trace amino acid metabolism in captive harbor seals (*Phoca vitulina*) through a two-year controlled feeding trial with either Pacific herring (*Clupea pallasi*) or walleye pollock (*Theragra chalcogramma*). Techniques were developed for measuring carbon and nitrogen isotope ratios of individual amino acids. Carbon and nitrogen trophic enrichments in serum of captive harbor seals varied with the two fish diets, which might have resulted from the changes in metabolic pathway due to the differing dietary protein intake between herring and pollock. Data on serum free amino acid compositions also showed, from a different perspective, that changes in seal metabolism occurred in response to these different feeding regimes.

Carbon and nitrogen isotopic composition of individual amino acids varied much more within an organism than across trophic levels, reflecting the distinct amino acid biosynthetic pathways. The similar patterns in relative amino acid carbon isotopic composition at different trophic levels indicated a conservative transfer of $\delta^{13}C$ from primary producers to top predators. Nitrogen trophic enrichments in different amino acids were not uniform, depending upon the extent to which a given amino acid was transaminated or deaminated, with several essential amino acids showing lesser variations than most non-essential and branched-chain amino acids. The differences in amino acid isotope ratios among phocids from the North Pacific or Atlantic and their counterparts from the Antarctic reflected the geographic variations in isotopic

composition of phytoplankton. The striking similarities in relative amino acid isotopic composition among phocids from the three distinct geographic locations indicated that phytoplankton worldwide had similar biosynthetic pathways during initial amino acid biosynthesis. This has important implications for using individual amino acid isotope ratios in studies of modern and prehistoric marine organisms.

Amino acid metabolic pathways governed the varying patterns of ^{15}N enrichments following ^{15}N-labeled amino acid tracer infusions. Tracer experiments further confirmed that phenylalanine, threonine, lysine and probably histidine may be useful as relatively conservative natural biomarkers. This study provided new insight into mechanisms of isotopic trophic dynamics in food web studies and improved our understanding of seal protein metabolism.

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The samples used in this study were authorized to the projects through several agencies. Samples from captive harbor seals were provided through EVOS project # /341 under Dr. M. Castellini and were collected under MMPA permit #881-1443 with UAF and ASLC-IACUC approval. Blood samples from the three species of Antarctic seals

were provided through a NSF Polar Programs grant, # OPP 9815176, to Dr. M. Castellini and were collected under MMPA permit #495-1524 with UAF-IACUC approval. Archived harbor seal blood samples from Prince William Sound were collected in 1994 under EVOS project #97001 under Dr. M. Castellini, in collaboration with EVOS project #/064 under K. Frost, Alaska Department of Fish and Game. Archived harbor seal blood samples from southeast Alaska were provided to Dr. M. Castellini by Alaska Department of Fish and Game. Blood samples from Atlantic harbor seals were collected through the Maine Department of Fish and Game, with assistance from Tamara Mau. Fish samples were provided through EVOS project #/341 under Dr. M. Castellini, and with the cooperation of the ASLC. The fish were food items for the marine mammals at the ASLC and stored under USDA inspection.

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Chapter 1

Introduction

Statement of the Problem

Analysis of naturally occurring stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) has become a powerful tool in the study of foraging ecology and animal physiology over the past three decades. A stepwise trophic enrichment of 3 -5 ‰ in nitrogen isotope ratios has been found from plants to herbivores to carnivores in terrestrial and marine ecosystems, in both field and laboratory studies (DeNiro and Epstein 1981a,b; Minagawa and Wada 1984; Schoeninger and DeNiro1984; Sealy et al. 1987; Owens 1987; Peterson and Fry 1987; Rau et al. 1992; Hobson and Welch 1992; reviewed by Michener and Schell 1994). Generally speaking, carbon isotope ratios are more conservative when they are transferred to higher trophic levels. Relatively small trophic enrichment (0 -1 ‰) along each level of the trophic ladder has made carbon isotope ratios an important tracer in distinguishing relative contributions of two distinct carbon sources in ecosystems, e.g., freshwater vs. marine, inshore vs. offshore, pelagic vs. benthic, etc. (Fry and Sherr 1984; Peterson et al. 1985; Dunton et al. 1989; Hobson et al. 1993; Fantle et al. 1999). This, on the other hand, makes carbon isotope ratios less useful in establishing the trophic status of an organism. However, a combination of carbon and nitrogen isotope ratios has proved very useful because co-variances of $\delta^{15}N$ with $\delta^{13}C$ can serve as good indicators of geographical migrations or prey switching (Schell et al. 1989; Saupe et al. 1989; Schell et al. 1998). Multiple stable isotope

approaches have been increasingly applied to a) delineate trophic structures of an ecosystem (reviewed by Michener and Schell 1994; Hobson et al. 1997); b) track seasonal migration patterns of marine mammals and foraging behavior of other species (Best and Schell 1996; Burton and Koch 1999; Harding and Stevens 2001); c) reconstruct paleodiet and paleoclimate from fossil remains (Sealy et al. 1987; Fogel et al. 1997; Hilderbrand et al. 1999) and d) assess animal physiology under a variety of conditions, such as starvation or fasting (Hobson et al.1993; Best and Schell 1996), nursing and weaning (Polischuk et al. 2001), and growth, aging or extreme environmental stress (reviewed by Ambrose1991).

Advantages of stable isotope techniques include the small sample size required, and the fact that minimally invasive sampling of fur, claws, or skin can provide useful information, which is especially important in live animal and valuable museum artifact studies. However, the precise mechanisms and related controlling factors that govern carbon and nitrogen isotope composition or fractionation are still poorly understood (Gannes et al. 1997). Large variations in both carbon and nitrogen isotope ratios have been documented in different ecosystems (marine vs. terrestrial); in different geographical locations (high latitude vs. tropical); interspecies (plants, herbivores, omnivores, carnivores); intraspecies (age, sex, habitats and seasons); in different tissue types (livers, blood, muscle, skin, keratinous tissues or bone collagen) or chemical fractions (protein, lipids, carbohydrates, etc.) within the same organism; and in different

physiological or nutritional states related to growth, maintenance, stress or fasting or food quality and availability.

Null Hypotheses and Objectives

The null hypotheses in this study are as follows:

1. Carbon and nitrogen isotope ratios of captive harbor seals accurately reflect diets under all conditions. Isotopic fractionation does not change in response to different diets or physiological conditions.
2. There are no essential amino acids in harbor seals and their prey species that can serve as conservative "biomarkers" of specific prey species or habitat usage.

To test the first hypothesis, a 2-year laboratory controlled feeding trial was conducted at the Alaska SeaLife Center in Seward, Alaska, from Sept. 1998 to Sept. 2000. Two captive harbor seals (*Phoca vitulina*) were fed known diets of either Pacific herring (*Clupea pallasi*) or walleye pollock (*Theragra chalcogramma*), which were switched every 4 months. One control harbor seal was fed a constant 1:1 mixed herring and pollock diet for the entire trial. Carbon and nitrogen isotope ratios in harbor seal serum and red blood cell proteins were monitored over the course of the feeding trial. Trophic enrichments were estimated by comparing the isotopic compositions in harbor seals and their known fish diet. Metabolic effects on isotopic fractionation were investigated in response to different fish diets and seasonal physiological changes.

A basic assumption in stable isotope food web analysis is that predictable relationships in isotopic composition exist between consumers and their diet. However, since ecological, biochemical and physiological processes may all result in isotope effects, the ultimate isotopic patterns in an organism are an integration of these multiple processes (Gannes et al. 1997). For example, one concern in applying stable isotope analysis to natural ecosystems is the possibility of consumers smoothing or amplifying isotopic signals through metabolic or/and physiological processes. Studies have shown that fasting or starvation may trigger protein sparing mechanisms in marine mammals that operate by regulating protein metabolic pathways (Castellini and Rea 1992). Highly enriched $\delta^{15}N$ values have been observed in rats, that responded to drought through physiological adaptations (Schoeninger and DeNiro 1984). Best and Schell (1996) have also observed significantly enriched $\delta^{15}N$ values in southern right whales during their winter breeding season in South African waters, when very little feeding is occurring, according to carbon isotope ratios. The effects of nutritional stress and environmental limitations on nitrogen isotope variations in terrestrial ecosystems have been widely investigated (reviewed by Ambrose 1991). The ability to reduce urinary water loss by increasing urine concentrations in response to water stress (Livingston et al. 1962; Maloiy et al. 1979) and the ability to restrict urea nitrogen excretion while on a low nitrogen diet (Ambrose 1986a, 1987) are well documented animal adaptations. Variations in the rate of urea excretion in response to differential nitrogen intake in humans are so straightforward that they have been recognized for decades (Epstein et al. 1957). As the

$\delta^{15}N$ value of urea is up to 10‰ more depleted than that of serum proteins, high rates of excretion of ^{15}N-depleted urea may result in large differences between diet and tissue $\delta^{15}N$ values (Sick et al. 1997). Therefore, metabolic pathways and physiological processes associated with protein intake or nitrogen balance in consumers may have important influences on the extent to which isotopic signatures of dietary protein are conserved in consumer tissue proteins (Gannes et al. 1997).

The use of laboratory controlled experiments to quantify isotopic fractionation by particular organisms and subsequent application of these data to infer trophic relationships in natural ecosystems is well established (DeNiro and Epstein 1978; 1981; Macko et al.1982; Tieszen et al. 1983; Minagawa and Wada 1984; Mizutani et al. 1991; Hobson and Clark 1992a, b; Fantle et al.1999). Harbor seals *(Phoca vitulina)* are small pinnipeds that are widely distributed throughout coastal regions of the Northern Hemisphere. The populations of harbor seals and several other marine mammal species in Alaska have declined significantly over the past two decades and are continuing the unexplained decrease (Pitcher 1990; Frost et al.1994; Small and DeMaster 1995). As apex predators in marine ecosystems, marine mammals play an important role of the so-called top-down control in community structures. Stable isotope techniques have been used increasingly to investigate the foraging ecology of seals and sea lions (Hobson et al.1996; Hobson et al.1997; Burton and Koch 1999; Hirons et al. 2000; Lesage et al., 2001; Kurle and Worthy 2001). However, the appropriate interpretation of field data in pinnipeds requires knowledge of both the isotopic fractionations that occur during

metabolic and physiological processes and the natural variations arising from changes in habitat usage or prey species. Due to the unique life cycles of marine mammals, laboratory controlled feeding trials using captive harbor seals as experimental models are particularly important. Controlled feeding trials can provide a unique opportunity to identify the physiological and biochemical processes affecting the isotopic fractionation and to elucidate the mechanisms that control the isotope patterns. A good understanding of the physiological and metabolic effects on isotopic fractionation between consumers and their diet is critical to validate the potential usage of stable isotope techniques in studies of wild mammals.

To test the second hypothesis on the presence of isotopically conservative amino acids, two ^{15}N-labeled amino acid tracer experiments were performed simultaneously with the controlled feeding trial. ^{15}N enrichments in individual amino acids were measured following the tracer infusions. The differential partitioning of nitrogen isotopes in transamination or deamination reactions of each amino acid was evaluated, and the relatively conservative "biomarkers" were identified.

Compound Specific Isotopic Analysis

Compound-specific isotope analyses (CSIA) have received increasing attention in recent years (Macko et al. 1987; Hare et al. 1991; Merritt and Hayes 1994; Fogel et al. 1997; Metges et al. 1996; Petzke et al. 1997; Metges and Daenzer 2000; Keil and Fogel 2001). The pioneering work by Abelson and Hoering (1961) revealed that photosynthetic

organisms exhibit substantial differences in the carbon isotope composition of individual amino acids when inorganic carbon is converted to living organic matter during photosynthesis. Varying nitrogen isotopic fractionation has been observed *in vitro* in microorganisms during transamination reactions via different metabolic pathways of amino acid biosynthesis (Macko et al. 1987). Further studies focusing on bone collagen demonstrated comparable but different patterns in amino acid carbon and nitrogen isotope ratios in herbivores and carnivores, including marine and terrestrial animals and both modern and fossil bone collagen (Hare et al. 1991; Fogel et al. 1997).

Amino acids either originate from diet or are synthesized *de novo*. Non-essential amino acids can be synthesized *de novo* by the organism, although some portion of these amino acids can also come from diet. Essential amino acids are those that cannot be biosynthesized by the organism itself, but must be supplied by dietary protein (Lehninger et al. 1993). Thus isotopic analysis of essential amino acids may provide dietary information that is not modified by metabolic or physiological processes. However, the isotopic composition of essential amino acids can be modified by oxidative breakdown, via transamination or deamination pathways, if there is an excess intake from diet. Amino acid catabolism plays a key role in major isotopic effects, since protein synthesis is usually an irreversible process without rate-limiting steps (Sick et al. 1997). Studies show that large isotopic effects occur if the precursor amino nitrogen is involved in transamination or deamination reactions (Macko et al. 1987; Sick et al. 1997). Both exogenous amino acids from dietary protein intake and the endogenous amino acids from

protein breakdown contribute to the precursor amino acid pool for new protein synthesis. As a result, the higher the proportion of the precursor amino acids that is metabolized to protein, the more similar the isotopic composition of newly-synthesized protein to that of the precursor amino acid pool. Therefore, if an amino acid comes exclusively from diet and most of it, either from recent dietary protein intake or endogenous protein breakdown, is used for protein synthesis, the isotopic signature of this amino acid would be conserved in the consumer tissue protein (Gannes et al., 1997).

As discussed earlier, the interactions among biochemical, physiological and ecological processes have made the isotope patterns in consumers so variable that unambiguous conclusions are often difficult to reach based solely on bulk stable isotope ratios of whole animal tissues (Gannes et al., 1997). If, however, we can identify one or a few essential amino acids whose isotope ratios are not modified by metabolic processes, then these essential amino acids can be used as "biomarkers" to track the specific prey species consumed or habitat usage. Combining isotopic analyses in individual amino acids of tissue proteins with conventional isotopic analyses in whole or bulk tissues may yield a more powerful tool for ecological and physiological research. For instance, naturally occurring carbon and nitrogen isotopic composition of individual amino acids may provide fine-scale information to the observed isotope variations in different tissue types or chemical fractions (O'Connell and Hedges 2001). Such data could help in tracing amino acid metabolic pathways, by providing insight into the differences in metabolic

effects on isotopic fractionation between essential amino acids and non-essential amino acids.

Methodology of Isotopic Analysis of Individual Amino Acids

Individual amino acid isotopic analyses have not been widely employed due to the technical difficulties in isolating pure, underivatized amino acids and collecting a sufficient amount of nitrogen and carbon for subsequent isotope ratio measurement. Basically there are two techniques that have been used: high performance liquid chromatography (HPLC) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Neither is completely satisfactory. The earliest method, which used standard liquid chromatography to isolate amino acids and identify each collected amino acid peak in a second analysis, followed by conventional IRMS, is very time-consuming and laborious (Abelson and Hoering 1961; Macko et al. 1987; Engel and Macko 1984; Hare et al. 1991). An alternative technique of GC-C-IRMS (Silfer et al. 1991; Demmelmair and Schmidt 1993; Metges et al. 1996; Macko et al. 1997; Metges and Petzke 1997; reviewed by Meier-Augenstein 1999) combines online GC separation and subsequent IRMS measurement, and is better in terms of the required time, labor, and sample amount, due to elimination of manual peak collection and sample preparation for collected fractions. Several different derivatization methods have been developed, especially for amino acid carbon isotope ratio measurements (Silfer et al. 1991; Demmelmair and Schmidt 1993; Metges and Petzke 1997). However, due to the need to form volatile amino acid derivatives for GC separation, which involves complex

derivatization procedures, isotopic fractionation during the reaction and changes in isotopic composition due to C or N added by the derivatization make this technique more complicated.

Modified techniques have been developed recently based on reversed-phase HPLC (van Eijk et al. 1997; O'Connell and Hedges 2001). They first use pre-column 9-fluorenylmethylchloroformate (FMOC-Cl) as a derivatization reagent, to achieve better detection, and then remove the derivative moiety prior to isotope ratio measurement. Although the reversed-phase HPLC technique is limited by relatively poor resolution and reproducibility of retention times compared to ion-exchange HPLC, it results in minimal isotopic fractionation effects on eluting peaks during amino acid separation (van Eijk et al. 1997; O'Connell and Hedges 2001). However, the procedures of pre-column derivatization and subsequent removal of the derivative moiety can cause some problems, e.g., difficulties in the recovery of individual amino acids after removing the derivative moiety and interference by excess derivatization reagents. Alternatively, detection of underivatized amino acids has been achieved using an evaporative light-scattering detector (ELSD), which minimizes the time needed for sample preparation (Alltech Associates, Inc.; Chaves-das-Neves and Braga-Morais 1997) since ELSD response does not depend on the optical characteristics of the samples (Alltech Associates, Inc.). The identical response for each amino acid reduces the inaccuracy in peak quantification.

In this study, a technique using semi-preparative reversed-phase HPLC separation of underivatized amino acids and subsequent IRMS measurement was developed based on the method of Schuster (1980). This technique avoids the pre- or post-column derivatization procedures that are required by most current methods. Since no derivatization is required and reversed-phase HPLC column separation is used, isotopic fractionation during amino acid separation is minimized. The gradient elution of amino acids using volatile organic and inorganic mobile phases has no influence on carbon or nitrogen isotope ratios of collected amino acid fractions after freeze-drying. As a result, carbon and nitrogen isotope ratios can be obtained from one HPLC run, because of relatively high column capacity and the high sensitivity of IRMS determination using a Finnigan Delta S isotope ratio mass spectrometer (Finnigan MAT, Germany).

Organization of the Dissertation

Chapter 1 has been a general introduction of the dissertation including a statement of the research problem and a brief review of current knowledge of the subject. Chapter 2 will describe the overall experimental design and analytical methods. Emphasis will be given to the method development and validation of the semi-preparative reversed-phase HPLC technique for isolating amino acids for subsequent IRMS measurement of carbon and nitrogen stable isotope ratios of individual amino acids. Chapter 3 will present the stable isotope data from a 2-year controlled feeding trial with captive harbor seals. Metabolic effects on isotopic fractionation will be discussed in relation to different diets of prey fish and seasonal physiological changes. Chapter 4 will address the natural

abundance of carbon and nitrogen isotopes in individual amino acids from different trophic levels of Prince William Sound and the adjacent Gulf of Alaska. The natural abundance of carbon and nitrogen isotopic composition of individual amino acids will also be compared among several seal species from the North Pacific, North Atlantic, and Antarctic. Chapter 5 will discuss the results of ^{15}N-enriched amino acid tracer infusion experiments on captive harbor seals. Data on changes in amino acid isotopic composition vs. time after infusion will be discussed based on a two-pool model. The isotopic effects of transamination or oxidative deamination reactions of amino acid metabolism will be explored by measurement of ^{15}N enrichments in different amino acids following the infusion of ^{15}N-enriched amino acid tracers. Chapter 6 will present data on amino acid compositions. Variations of serum free amino acids in captive harbor seals will be discussed in the context of varying dietary protein intake. Serum free and protein hydrolysable amino acid compositions of wild harbor seals from the North Pacific and Weddell, Ross and crabeater seals from the Antarctic will be compared. Chapter 7 will summarize isotopic data for the two fish diets used in the captive feeding study (Chapter 3): Pacific herring and walleye pollock caught in Prince William Sound or the Gulf of Alaska. The effects of proximate composition, locations and year of capture, and fish standard length or mass on carbon and nitrogen isotope ratios of fish carcasses will be discussed. Chapter 8 will present data on the seasonal growth patterns of harbor seal vibrissae, elucidated using ^{15}N-labeled isotope tracers. Chapter 9 will summarize the main conclusions of this dissertation and offer suggestions for future study.

Chapter 2

Experimental Design and Methods

Controlled Feeding Trial

A 2-year controlled feeding trial was conducted from 15 Sept. 1998 to 15 Sept. 2000 at the Alaska SeaLife Center (ASLC) in Seward, Alaska, with four adult captive harbor seals identified as Snapper, Pender, Travis and Poco. The harbor seals were housed in outdoor tanks and fed herring for three months before the controlled feeding trial started. Pacific herring (*Clupea pallasi*) and walleye pollock (*Theragra chalcogramma*) were utilized in the feeding trial as representative prey species of harbor seals. These two species have been hypothesized to represent two potentially different nutritional regimes affecting survival of wild seals and sea lions. Seals usually consumed 5 % ~10 % of their body mass each day and they ate more pollock than herring.

Table 2.1 shows the feeding schedule for the four captive harbor seals. One feeding cycle was defined as a 4-month feeding period, and there were a total of six feeding cycles. Snapper, a 15 year old male, was fed on a mixed diet of 1:1 herring and pollock as a control for the entire trial. Pender and Travis, which were both 2.5 years old, were first fed herring, then pollock, then switched to the alternative diet every 4 months. Poco, a 23 year old female, was fed pollock initially and switched to the alternative diet at the same time as Pender and Travis, but in reverse order to compensate for seasonal effects.

Table 2.1 Feeding schedule of captive harbor seals during the two-year controlled feeding trial. Specific batches of fish diets are indicated in parentheses.

Starting date	15 Sept 98	16 Jan 99	01June 99	16 Sept 99	16 Jan 00	16 May 00
Ending date	15 Jan 99	15 May 99	15 Sept 99	15 Jan 00	15 May 00	15 Sept 00
Feeding cycle	1	2	3	4	5	6
Pender	Herring	Pollock	Herring	Pollock	Herring	Pollock
Travis	(H4)	(P3)	(H4)	(P3)	(H4)	(P5)
Poco	Pollock	Herring	Pollock	Herring	Pollock	Herring
	(P2)	(H4)	(P3)	(H4)	(P3)	(H6)
Snapper	Mixed diet: 50% herring and 50% pollock					
	H4--P2	H4--P3	H4--P3	H4--P3	H4--P3	H6--P5

^{15}N-labeled Amino Acid Tracer Experiments

Two ^{15}N-labeled amino acid tracer experiments were performed during the controlled feeding trial, with the goal of identifying any essential amino acids in harbor seals that could act as conservative biomarkers of specific prey species or habitat usage. Amino acids enriched with ^{15}N (95-99%) (Cambridge Isotope Laboratories, Inc.) were prepared in sterile saline solution, sealed in ampules and autoclaved for 25 minutes. The concentrations of different amino acids in the injection solution were based on their solubility and were 100 mg/ml for glycine, 52.24 mg/ml for valine, 26.83 mg/ml for phenylalanine and 20.99 mg/ml for leucine. The total amount of each amino acid infused was approximately 0.5 g for glycine and 1g for leucine, valine and phenylalanine per seal, which proved to be sufficient to track the label. All the procedures were coordinated with other ongoing harbor seal projects, approved by the ASLC scientific committee, and conducted as required by the Institutional Animal Care and Use Committees of the University of Alaska and ASLC.

Table 2.2a summarizes the details of the two ^{15}N-labeled amino acid tracer experiments. Harbor seal vibrissae sampling information is given in Table 2.2b. During the first amino acid tracer experiment, the ^{15}N-labeled amino acid glycine was infused intravenously on 8 December 1998 into Snapper and Poco and on 16 December 1998 into Pender. The essential amino acids leucine, valine and phenylalanine were infused, respectively, into Snapper, Poco and Pender on 22 June 2000 during the second amino acid tracer experiment. Harbor seal Travis served as a label control and no ^{15}N-labeled

Table 2.2a ^{15}N-labeled amino acid tracer experiments

Tracer Infusion	Dosage*	Seals	Experiment Period	Duration (days)
	Glycine	Pender	16 Dec 98-15 Sep 99	275
1st experiment in	Glycine	Poco	8 Dec 98-15 Sep 99	282
Dec 1998	Glycine	Snapper	8 Dec 98-15 Sep 99	282
	Phenylalanine	Pender	21 June 00-5 Sep 00	77
2nd experiment in	Valine	Poco	21 June 00-5 Sep 00	77
June 2000	Leucine	Snapper	21 June 00-5 Sep 00	77

* A total of approximately 1 g ^{15}N-labeled amino acid tracers was infused.

Table 2.2b Sampling information for captive harbor seal vibrissae

Seals	Sex/Age (yrs)	Sampling Date	Days after Injection	Whisker Length (cm)	Tracer Type
Pender	Male/2.5	19 Dec 98	4	8.8	^{15}N-glycine
		25 Feb 99	72	3.8	^{15}N-glycine
		20 May 99	156	2.0	^{15}N-glycine
		16 Sept. 99	275	10.0	^{15}N-glycine
		5 Sept. 00	77	6.3	^{15}N-phenylalanine
Poco	Female/23	8 Dec 98	0	10.0	^{15}N-glycine
		18 May 99	162	6.2	^{15}N-glycine
		15 Sept. 99	282	10.0	^{15}N-glycine
		5 Sept. 00	77	6.0	^{15}N-valine
Snapper	Male/15	8 Dec 98	0	10.0	^{15}N-glycine
		15 Sept. 99	282	10.0	^{15}N-glycine
		5 Sept. 00	77	6.0	^{15}N-leucine

amino acid tracer was injected. Carbon and nitrogen isotope ratios in Travis reflect the natural variations in response to changes in diet or metabolic or physiological processes.

Harbor Seal Blood Sampling and Stable Isotope Analysis

Blood samples were collected at two-week intervals following an overnight fast. Serum (no additives was added in sample tubes) and unclotted red blood cells (RBCs) were separated by centrifugation immediately after blood collection and frozen at –80° C. Care was taken to avoid contamination of the serum with leukocytes. Since RBCs were not washed, some retention of serum occurred. During the amino acid tracer experiments, blood sampling was conducted at 4 hrs, 1 day, 2 days and 3 days following the infusion of amino acid tracers to track the transfers of the label. Serum and RBC samples were freeze-dried and ground to a powder for homogeneity. The isotope ratios of carbon and nitrogen were measured with a Europa 20/20 continuous flow isotope ratio mass spectrometry system. Results are reported using standard δ notation in parts per thousand (‰) relative to Pee Dee Belemnite (VPDB) for carbon and atmospheric N_2 for nitrogen. Analytical precision of peptone standards was ±0.2‰ for both carbon and nitrogen.

Diet Fish Sampling and Analysis

Five batches of Pacific herring and four batches of walleye pollock were analyzed. Pacific herring Batch 1 (H1) was caught in Prince William Sound (PWS), Alaska (AK) during November 1997. Batch 2 (H2) was from the Atlantic Ocean. Batch 4 (H4) was caught in PWS, AK during November 1998 and Batch 6 (H6) was caught near

Petersburg, AK during December 1999. Herring Batch 5 (H5) was not used in the feeding trial due to the small size of the juvenile bait herring and the unknown capture location. Walleye pollock Batch 2 (P2) was caught in the Gulf of Alaska (GOA), AK during March 1998, Batch 3 (P3) was also from the GOA, AK and was caught in January 1999, and Batch 4 (P4) was caught near Cordova, AK during March 1999. Pollock Batch 5 (P5) was donated by private party and the catch location and time were unknown. Five batches of two fish species were used in the feeding trial. H4 was used in the controlled feeding trial from Nov. 98 to May 00, H6 from June 00 to Sept. 00, P2 from Sept. 98 to 15 Feb. 99, P3 from 16 Feb. 99 to May 00 and P5 from June 00 to Sept. 00.

Fresh frozen fish specimens were obtained from various commercial fish processing companies. The fish had been individually ice-glazed and remained frozen until they were processed for proximate analysis. Standard body length and mass of each specimen was measured before processing. A small portion of homogenized and freeze-dried whole fish tissues was used for stable isotopic analysis as described above. Lipid-free fish tissues were obtained using a lipid extraction procedure adapted from Bligh and Dyer (1959).

Harbor Seal Vibrissa Sampling and Stable Isotope Analysis

Vibrissae were clipped from the muzzle of captive harbor seals at intervals during the controlled feeding trial and especially after ^{15}N-labeled amino acid tracer infusions (Table 2.2a). Vibrissae were cleaned with steel wool and sectioned at ~2mm intervals

from base to tip for isotopic analysis. Other samples, such as fur and nails, were taken opportunistically and washed prior to analysis.

Amino Acid Analysis Protocols

Standard and reagents

Amino acid composition analysis using pre-column o-phthaldialdehyde (OPA) derivatization and subsequent separation on a HPLC reversed phase column combined with UV detection was adapted from several published methods (Lindroth and Mopper 1979; Hill et al. 1979; Henrichs and Williams 1985; Mopper and Dawson 1986). Individual standard L-amino acids and the AA-S-18 amino acid standard mixture containing 18 amino acids (all from Sigma Chemical Co.) were used for identification and quantification, respectively. Standard L-amino acids were dissolved individually in 0.01 M HCl and kept at -20°C. An internal standard of 1.5 μmol/ml α-amino adipic acid (Sigma) was used for quality control. All reagents used were analytical grade or above. All solvents were HPLC grade. All water used was distilled and processed using a Milli-Q system.

Deproteinization of serum for free amino acid measurement

Sulfosalicylic acid (SSA) was used to precipitate protein from serum samples prior to free amino acid measurement. Serum samples were brought to room temperature and 0.4ml serum was transferred to a microfuge tube and 60 μl 20% SSA added. After mixing vigorously and cooling on ice for 10 minutes, the solution was centrifuged to

pellet the precipitated protein and the supernatant filtered through a 0.2 μm POLYPURE syringe filter before HPLC analysis.

Hydrolysis

A standard acid hydrolysis procedure for amino acid analysis of biological samples was used. Approximately 20 mg of dried harbor seal serum and red blood cells or fish tissues were hydrolyzed in a PTFE-capped Pyrex vial under an atmosphere of N_2 in 2 ml of 6N HCl for 22 hrs at 110 °C. Protein hydrolyzates were then vacuum evaporated to dryness at 60°C and re-dissolved in 0.01 M HCl and filtered with a 0.2 μm POLYPURE syringe filter prior to HPLC injection.

Derivatization procedure

An appropriate volume of a free or hydrolyzed amino acid sample was added to a 1.8 ml autosampler vial with 1.2 ml of 0.025 M borate buffer (pH=10.4). An internal standard of 50 μl α-amino adipic acid and 50 μl of derivatization reagent for derivatization were subsequently added and reacted for exactly 2 min prior to HPLC injection. The derivatization reagent was prepared daily from 20 mg OPA, 40 mg sodium dodecyl sulfate (SDS) and 24 μl 2-mercaptoethanol dissolved in 2 ml methanol and stored in the dark. Borate buffer of pH=10.4 was prepared by adding 22.7 ml of 0.1 M NaOH and 50 ml of 0.025 M Borax (sodium tetraborate) to 27.3 ml distilled water to obtain a final volume of 100 ml.

High performance lipid chromatography (HPLC)

The HPLC system consisted of Alltech model 526 dual piston pumps, a model 570 autosampler and a model 530 column heater. OPA amino acid derivatives were detected at 340 nm by a Linear model UVIS 200 UV detector. The HPLC system was controlled externally by a PENELSON NCI 900 network chromatography interface. Data were acquired on a personal computer running Microsoft Windows 98 using TURBOCHROM software (Perkin-Elmer). Amino acid separation was achieved on an Allsphere ODS-II column (250×4.6 mm, 5 μm particle size). The mobile phase gradient was run from an initial 15% solvent A (methanol) and 85% solvent B to 70% solvent A using a linear gradient over 23 minutes, held at 70% solvent A for 5 minutes, then returned to the initial composition over 2 minutes and held another 5 minutes before the next injection. Mobile phase solvent B was 0.025 M phosphate buffer (pH=6.5, 1:1 Na_2HPO_4 and NaH_2PO_4) containing 3% tetrahydrofuran (THF). The flow rate was 1.4 ml/min. Under these conditions, all amino acids except glutamine and histidine could be resolved consistently. Typical chromatograms are shown in Fig. 2.1a,b.

Quality control

The peaks were identified by retention time of individual standard amino acids and response factors for individual amino acids were measured using the AA-S-18 amino acid standard mixture. The AA-S-18 standard calibration was run twice before starting the sample analysis and after every seven samples. An internal standard, α-amino adipic acid, was added to each sample before chromatography. Analytical precision was

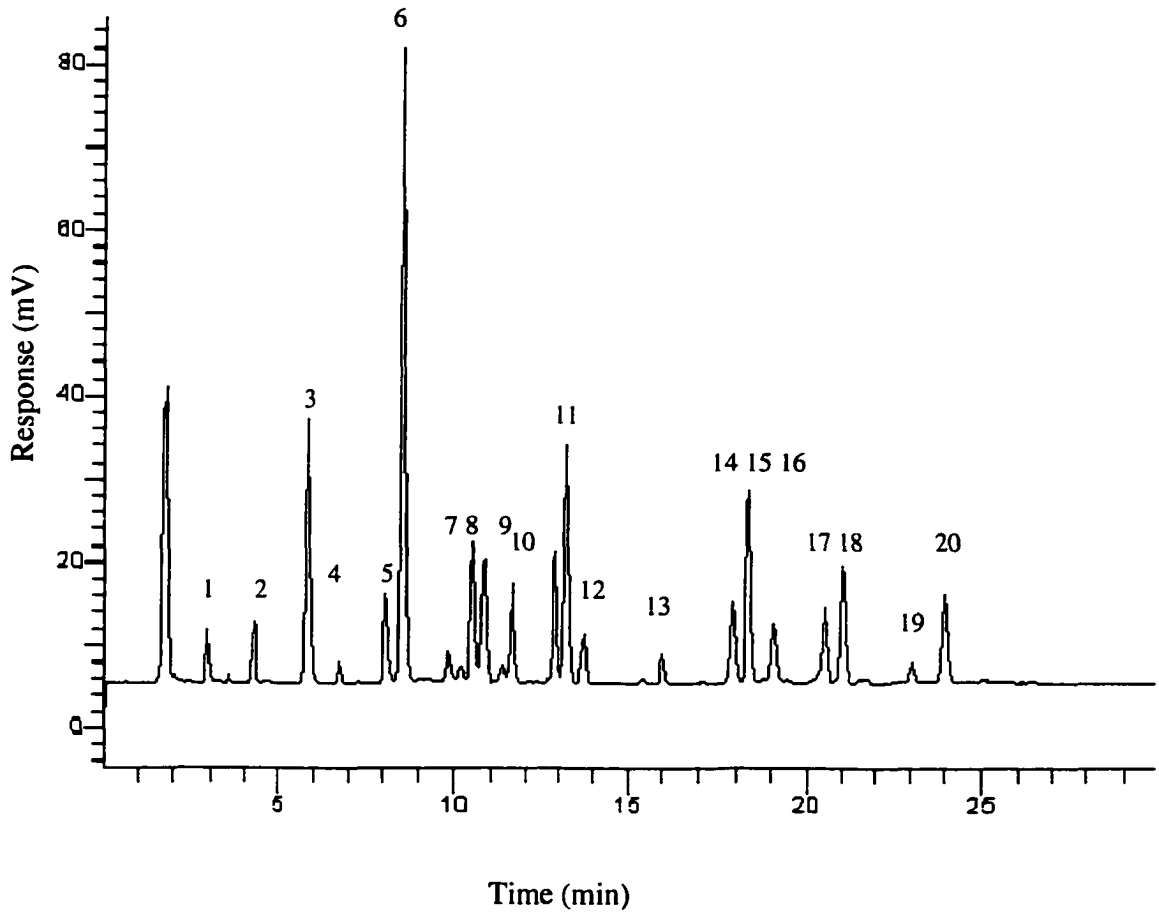


Fig. 2.1a A typical chromatogram of free amino acids in harbor seal serum using the pre-column OPA method. 1. Asp 2.Glu 3.ISTD 4.Asn 5.Ser 6.His (+Gln) 7.Gly 8.Thr 9.Arg 10.Tau 11.Ala 12.Tyr 13.α-ABA 14.Met 15.Val 16.Phe 17.Ile 18.Leu 19.Orn 20.Lys. ISTD: α- amino-adipic acid. Refer to amino acid abbreviations in Table 2.3. The chromatographic conditions were those described in the text.

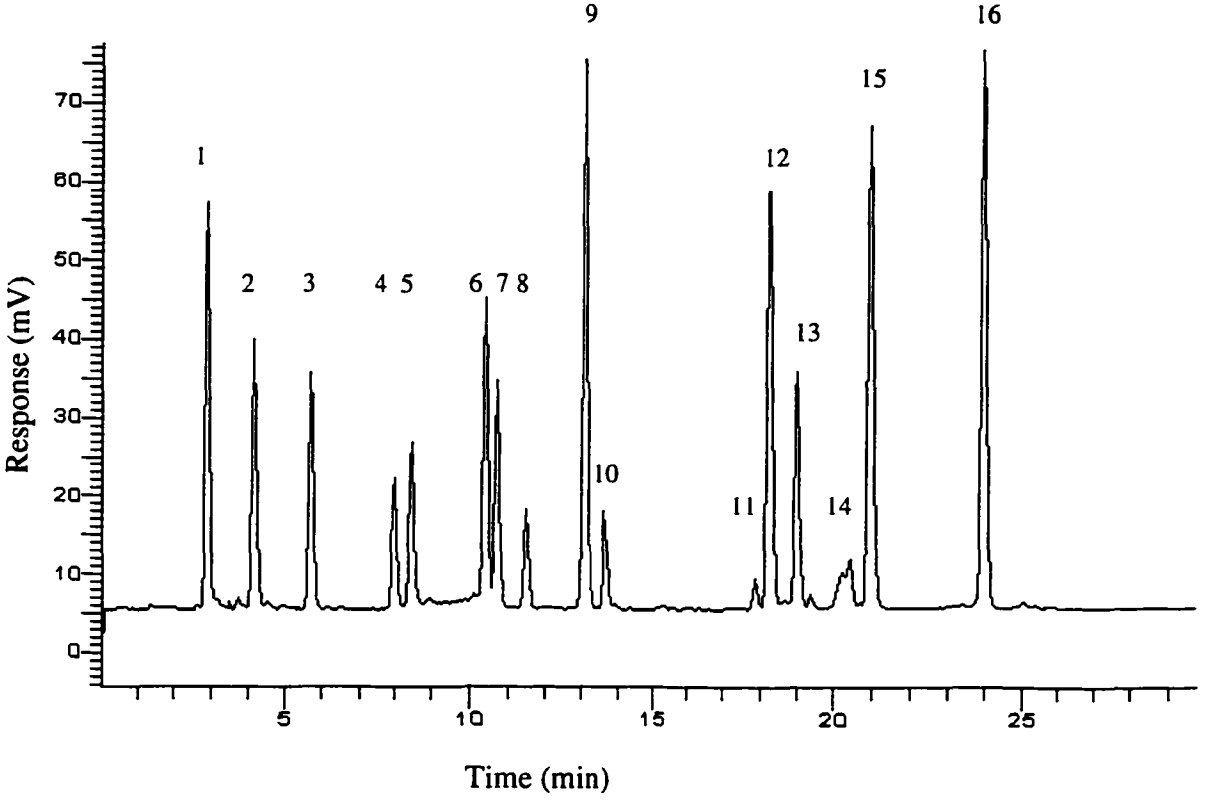


Fig. 2.1b A typical chromatogram of hydrolyzable amino acids in harbor seal red blood cells using the pre-column OPA method. 1. Asp 2.Glu 3.ISTD 4.Ser 5.His 6.Gly 7.Thr 8.Arg 9.Ala 10.Tyr 11.Met 12.Val 13.Phe 14.Ile 15.Leu 16.Lys. ISTD: α- amino-adipic acid. Refer to amino acid abbreviations in Table 2.3. The chromatographic conditions were those described in the text.

assessed by 6 consecutive runs of the AA-S-18 standard and the coefficients of variation for peak areas ranged from 0.72% to 3.36% as shown in Table 2.3. A linear relationship between the concentration and peak area of each amino acid was found by analyzing the standard amino acid mixture at concentrations ranging from 10 to 100 μmol/l. The linear regressions of all identified amino acids showed satisfactory coefficients of correlation (r^2 =0.995~0.999).

Amino acid composition notation

The amino acid data are reported using the following notation. Amino acid concentrations in protein hydrolyzates (hydrolysable, HAA) are expressed as μmol per gram of dry serum or red blood cells (μmol/g, dry weight). HAA concentrations of serum are also converted to μmol per liter in volume concentrations (μmol/l). A total of 15 hydrolysable amino acids were measured and the sum of 14 of these is reported as the total hydrolysable amino acid pool (THAA). (Methionine is not included due to the partial loss by hydrolysis). Serum free amino acid (FAA) concentrations are reported as μmol per liter (μmol/l) and a total of 20 free amino acids were analyzed; 19 concentrations are reported, since histidine co-elutes with glutamine. In this study, histidine (His), isoleucine (Ile), leucine (Leu), valine (Val), lysine (Lys), phenylalanine (Phe) plus tyrosine (Tyr), threonine (Thr), methionine (Met) and arginine (Arg) are defined as essential amino acids (EAA) for harbor seals. The remaining amino acids, alanine (Ala), glycine (Gly), serine (Ser), asparagine (Asn), aspartic acid (Asp) (Asn + Asp = Asx), glutamine (Gln) and glutamic acid (Glu) (Gln + Glu = Glx) are defined as

Table 2.3 Reproducibility of peak area response factors of standard amino acids for the pre-column OPA derivatization method

Amino Acid	Abbreviation	Mean response factor (area / pmol) (n=6)	SD	CV (%)*
Alanine	Ala	241	2.24	0.93
Glycine	Gly	215	5.20	2.42
Serine	Ser	243	1.74	0.72
Aspartic acid	Asp	224	2.98	1.33
Glutamic acid	Glu	221	2.40	1.09
Arginine	Arg	246	3.04	1.24
Histidine	His	173	2.01	1.16
Isoleucine	Ile	299	8.29	2.77
Leucine	Leu	247	2.35	0.95
Valine	Val	254	2.58	1.02
Lysine	Lys	345	11.58	3.36
Phenylalanine	Phe	232	2.12	0.91
Tyrosine	Tyr	201	0.99	0.82
Threonine	Thr	233	2.38	1.02
Methionine	Met	245	2.33	0.95
Asparagine	Asn	236	5.34	1.88
Taurine	Tau	252	7.08	2.89
Ornithine	Orn	309	3.32	0.80
α-amino butyric acid	a-ABA	244	5.42	1.30

*Coefficient of variation was calculated as (Standard deviation/ Mean) x 100.

non-essential amino acids (NEAA). The sum of 9 amino acids (His, Ile, Leu, Val, Lys, Phe +Tyr, Thr, Arg) is reported as the essential amino acid pool for HAA and the sum of Ala, Gly, Ser, Asx and Glx as the non-essential amino acid pool. For the serum free amino acid composition, Met is added to the EAA pool and His is omitted from the EAA pool due to its co-elution with Gln. Non-essential serum free amino acids (Ala, Gly, Ser, Asn, Asp, Gln+His and Glu) are reported as the NEAA pool. All 20 free amino acids, including taurine (Tau), ornithine (Orn) and α-amino butyric acid (α-ABA), are reported as the total serum free AA pool (TFAA).

Stable Isotope Analysis of Individual Amino Acids

RP-HPLC methodology and scale-up

Reversed-phase high performance liquid chromatography (RP-HPLC) has been widely used in amino acid analysis with the help of either pre- or post-column derivatization, which improves both the selectivity and sensitivity of detection (reviewed by Hancock and Harding 1984). The most successful method using RP-HPLC with an UV detector to measure amino acids without any derivatization procedures was reported by Schuster (1980). Unlike the non-polar reversed-phase columns commonly employed in separation of amino acid derivatives, a polar amino (NH_2) column is used to achieve the separation of amino acids, instead of their derivatives. This column has proven to be particularly good for polar amino acids that were in general only slightly retarded on non-polar columns, such as C8 or C18. The polar amino column has a substantially different order of elution times: hydrophobic < polar uncharged < basic < acidic

(Schuster 1980; Hancock and Harding 1984). Due to the extraordinary advantage of not requiring derivatization in terms of the subsequent stable isotopic measurement, this method was used on both analytical and semi-preparative scales with acceptable resolution and reproducibility. Fig. 2.2 shows a typical chromatogram of a harbor seal serum sample on a semi-preparative scale. Scale-up on a semi-preparative column did not diminish resolution significantly. Pilot experiments showed that an injection of 5 mg of protein hydrolyzates was necessary to obtain a sufficient amount of amino acid nitrogen and carbon for the subsequent isotopic analysis. A semi-preparative column with a 250 x 10 mm I.D. proved sufficient for the isolation and stable isotopic analysis of all amino acids.

Amino acid separation for mass spectrometry was achieved on Econosphere NH_2 column (Alltech Associates, Inc.) by a gradient elution (Table 2.4) at a flow rate of 4 ml/min at 38°C column temperature. The mobile phase solvent A was acetonitrile/water (500:70 v/v) and solvent B was 0.01 M potassium dihydrogen phosphate (pH = 4.3, adjusted with phosphoric acid). A column with a 250 x 4.6 mm I.D. (5 μm particle size) and a 20 μl sample loop was used for analytical scale separation and a 250 x 10 mm I.D. (10 μm particle size) and a 500 μl sample loop for semi-preparative scale. The data channel of the UV detector was set at a wavelength of 210 nm. The amino acid peaks were collected using a fraction collector. After HPLC column separation, the eluted amino acid fractions were first evaporated under a stream of air to remove most of the acetonitrile and water, then lyophilized and weighed into a tin cup. Carbon and nitrogen

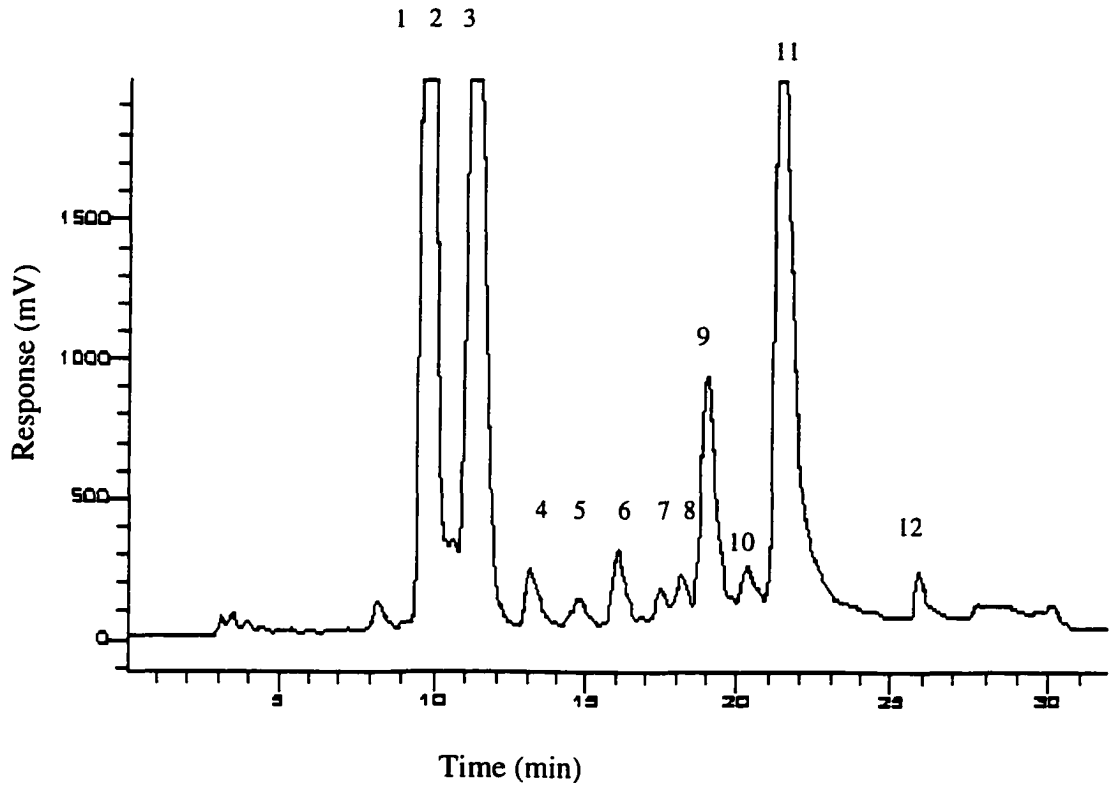


Fig. 2.2 A typical chromatogram of amino acids in a harbor seal serum sample using a semi-preparative NH_2 column. 1. Phe 2.Ile/Leu 3.Tyr 4.Val 5.Pro 6.Ala/Thr 7.Gly 8.Ser 9.Arg 10.Lys 11.His 12.Glu. Refer to amino acid abbreviations in Table 2.3. The chromatographic conditions were those described in the text.

Table 2.4 Mobile phase gradient elution program for NH_2 column

Time (minute)	Solvent A [a] (%)	Solvent B [b] (%)
0	90	10
5	90	10
20	70	30
27	50	50
32	90	10
37 (column conditioning)	90	10

a. Acetonitrile : water (500:70 v/v)

b. 0.01M potassium dihydrogen phosphate (KH_2PO_4, pH = 4.3)

isotope ratios of collected amino acids were measured with a Finnigan Delta S isotope ratio mass spectrometer (Finnigan MAT, Germany). Results are reported using standard δ notation in parts per thousand (‰) relative to VPDB for carbon and atmospheric N_2 for nitrogen. Analytical precision of peptone standards is ±0.3‰ for both carbon and nitrogen isotope ratios. The standard deviation for duplicate isotopic analysis of individual amino acids in harbor seal serum protein hydrolysates ranged from 0.3 to 2.55%. Amino acid isotope ratios are not reported for methionine, due to partial loss during acid hydrolysis. Glx is used to represent glutamate plus glutamine because of the complete conversion of glutamine to glutamic acid. Similarly, Asx represents aspartic acid plus asparagine.

Effects of solvents and buffer salts on isotope ratio measurements

Although the acetonitrile used in the HPLC mobile phase was easily removed by evaporation, tests were conducted to compare the isotope ratios among the standard amino acids, the standard amino acids lyophilized from acetonitrile/water (80/20) and the standard amino acids lyophilized from a mixture of phosphate buffer and hydrochloric acid (Table 2.5). No significant differences were found among the procedures, indicating that mobile phase solvents (acetonitrile and buffer salt solution) mixed with the collected amino acids did not affect the subsequent isotope ratio measurements.

Isotopic fractionation during RP-HPLC column separation

Considering that the column separation processes may result in isotopic fractionations in eluted amino acid peaks, some amino acid isotope ratios were compared with standard amino acids before and after column separation (refer to Table 2.5). Most runs showed identical amino acid isotope ratios in collected amino acid peaks after RP-HPLC column separation, when amino acids were completely separated and collected. However, a few amino acid isotope ratios such as those Leu/Ile pairs and Ala/Thr pairs were found to be different due to the partial co-elution with nearby peaks. Also, shifts in retention time and increasing co-elution of peaks were observed with the aging of the column. Therefore, a purity test of each collected peak was required to determine to what extent the nearby peaks overlapped and the effects on the amino acid isotope ratios.

Table 2.5 Comparison of isotope ratios in standard amino acids after separation using the NH_2 column and the effects of acetonitrile and mobile phase buffer salts

AA	Solid standard AA		Standard AA in 10% 20 mM phosphate buffer and 90% 0.01M HCl		Standard AA in Acetonitrile/H_2O (90:10)		Standard AA collected from NH_2 column				
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	SD	$\delta^{15}N$	SD	N
Thr	-8.17	-9.49			-8.56	-9.95	-6.14	0.06	-11.50	0.19	4
Ala	-6.75	-22.46			-7.09	-22.94					
Gly	11.28	-36.56	10.85	-35.99	10.82	-36.45	10.90	0.06	-36.07	0.34	7
Ser	2.22	-30.24	1.84	-30.18	2.46	-30.15					
His	-10.20	-9.77	-10.22	-10.34	-10.28	-10.19	-9.04		-13.83		2
Lys	0.90	-11.90			0.68	-12.04					
Arg	-7.80	-14.12	-8.02	-14.19	-7.68	-15.56	-6.49	0.74	-14.46	2.23	5
Val	-6.60	-10.81	-6.91	-11.23	-6.79	-11.67	-6.25	0.61	-11.83	0.22	4
Pro	-8.44	-12.34			-8.82	-12.73	-7.36	0.39	-13.66	0.59	4
Met	1.43	-31.44	1.26	-31.53	1.84	-31.48	2.10	0.10	-31.50	0.11	4
Ile	-8.00	-11.89	-8.07	-12.30	-7.88	-17.16					
Leu	-0.33	-27.96			-0.87	-28.26	0.25		-28.44		2
Phe	1.58	-10.76	1.37	-11.24	1.56	-11.39	2.39		-11.30		2

Purity verification by quantification of amino acids in each eluted peak

Pre-column OPA amino acid analysis, as described above, was conducted to verify the purity of each collected peak. Results showed that the contamination between nearby peaks was minimal if peaks were baseline resolved. In addition, OPA analyses of peaks collected with or without delay time were compared, to test whether or not the theoretical delay time of fraction collector in our HPLC system was appropriately set up.

Separation of non-polar amino acids

Accurate measurement of individual amino acid isotope ratios using the no derivatization procedure and UV detection at a short wavelength was difficult because of the highly variable molar response factors for different amino acids. Several amino acids, such as Leu and Ile, with low molar response factors, were usually overlapped by nearby aromatic amino acids, such as Phe and Tyr, with high molar response factors. To insure complete separation, the five amino acid components (Phe, Leu, Ile, Met and Tyr) were collected after separation on the NH_2 column, concentrated, and run again using a non-polar Alltima C18 column (250x10 mm I.D., 5 μm particle size). The mobile phase solvent A was acetonitrile/water (500:70 v/v) and solvent B was 0.01 M potassium dihydrogen phosphate (pH ≈ 2.5 adjusted using phosphoric acid). The mobile phase gradient elution program was as follows: an initial condition of 3% solvent A and 97% solvent B for 10 minutes, then change solvent A from 3% to 30% with a linear gradient during next the 10 minutes, return to the initial condition 1 minute, and hold for 5 minutes for column conditioning before the next injection. Good resolution was

achieved for all five non-polar amino acids under the above conditions, especially for Leu and Ile, which were very difficult to resolve on the NH_2 column. Moreover, the peak resolution was sufficient to avoid using ion-pairing reagents such as trifluoroacetic acid (TFA), which is commonly used to improve resolution and peak shapes in reversed-phase HPLC. Fig. 2.3 shows a typical chromatogram of these five components in a harbor seal serum sample without TFA. Data in Table 2.6 show the effects of TFA in mobile phase solvents on the nitrogen isotope ratios, indicating that use of this additive must be avoided. The nitrogen isotopic composition changes by TFA might be caused by its ability to form micelles structures.

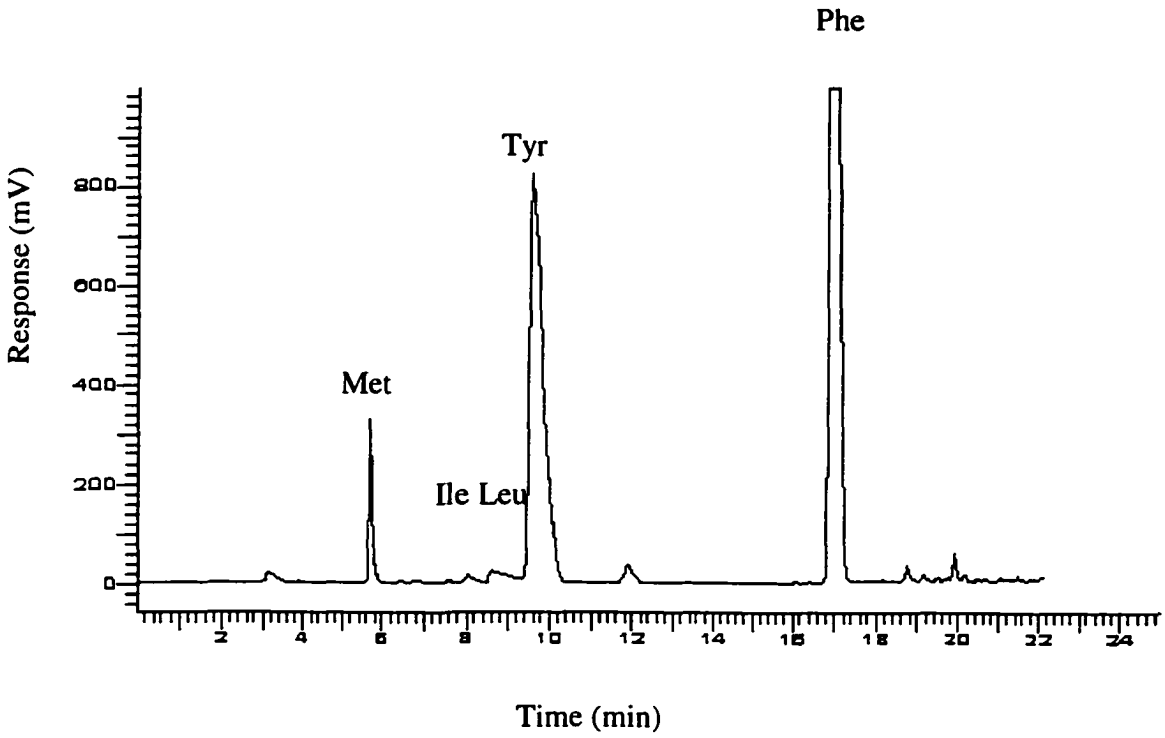


Fig. 2.3 A typical chromatogram of five non-polar amino acids separated from a harbor seal serum sample using a semi-preparative Alltima C18 column.

Table 2.6 Comparison of isotope ratios in non-polar standard amino acids after separation using the Alltima C18 column and the effect of TFA on nitrogen isotope ratios

Amino Acids	Initial standard AA		Standard AA dissolved in water containing 0.1% TFA		Collected standard AA from Alltima C18 column	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Val	-6.60	-10.81	-6.64	-13.34	-7.30	-11.83
Pro	-8.44	-12.34			-7.51	-14.06
Met	1.43	-31.44	1.31	-31.84	1.30	-30.92
Ile	-8.00	-11.89	-7.83	-16.96	-8.71	-12.84
Leu	-0.33	-27.96			-0.60	-28.18
Phe	1.58	-10.76	1.83	-14.22	1.81	-11.50

Chapter 3

Stable Isotope Ratios in Captive Harbor Seals (*Phoca vitulina*): Response to Diet and Metabolic Changes

ABSTRACT

A critical assumption in using stable isotope analysis in studies of foraging ecology is that a predictable relationship in isotopic composition exists between consumers and their diets. We tested this assumption on captive harbor seals (*Phoca vitulina*) through a two-year controlled feeding trial, with two representative fish diets of either exclusively Pacific herring (*Clupea pallasi*) or walleye pollock (*Theragra chalcogramma*), which were switched every 4 months. One control harbor seal was fed a constant 1:1 herring and pollock mixed diet. Carbon and nitrogen isotope ratios in harbor seal serum and red blood cells (RBCs) were monitored to investigate the metabolic effects on isotopic fractionation in response to different fish diets and seasonal physiological changes. Carbon and nitrogen isotope ratios in different tissue types of seals were also compared, with a goal of elucidating how chemical composition of tissue proteins may affect tissue-specific isotope ratios. Results show that $\delta^{13}C$ signatures in harbor seal serum tracked diet switches between herring and pollock. However, harbor seals fed on a herring diet fractionated carbon to a greater extent than those fed on pollock. $\delta^{13}C$ signatures in RBCs varied much less in response to dietary changes. $\delta^{13}C$ values in RBCs were, overall, more enriched than those in serum. A mean carbon trophic enrichment of 4.18‰ was found for harbor seal serum on a herring diet (n=62), compared

with 2.35‰ on a pollock diet (n=67). The control seal fed on a constant 1:1 mixed diet had an intermediate mean carbon trophic enrichment of 3.36‰ (n= 51).

$\delta^{15}N$ signatures in harbor seal serum co-varied with $\delta^{13}C$ as the diet switched, although there was no statistically significant difference in $\delta^{15}N$ values between herring and pollock. In contrast to carbon, harbor seals fed a pollock diet fractionated nitrogen to a slightly greater extent and tended to have higher $\delta^{15}N$ values in their serum proteins than those fed a herring diet. The mean nitrogen trophic enrichment in serum was 3.83‰ on the herring diet (n=18) and 4.55‰ on the pollock diet (n=22). By comparing harbor seal daily body protein intake between herring and pollock diets, we see evidence that differing protein metabolic pathways corresponding to the different dietary protein intake may be responsible for these differences in isotopic fractionations. Lipid effects may also account for some of the differences in carbon isotopic fractionation.

Keywords: $\delta^{13}C$; $\delta^{15}N$; controlled feeding trial; captive harbor seals; metabolic and physiological processes; isotopic fractionation.

INTRODUCTION

A basic premise of using stable isotopic techniques in studies of foraging ecology is that there is a predictable relationship in isotopic composition between consumers and their diets. Use of laboratory controlled experiments to quantify isotopic fractionation of particular organisms and subsequent application of these data to infer trophic

relationships in field studies is a well-established approach (DeNiro and Epstein 1978; 1981; Macko et al.1982; Minagawa and Wada 1984; Mizutani et al. 1991; Hobson and Clark 1992a, b; 1993; Fantle et al.1999). A variety of applications using stable isotopic techniques in pinnipeds have been reported (Hobson et al.1996; Hobson et al.1997; Burton and Koch 1999; Hirons et al. 2000; Lesage et al. 2001; Kurle and Worthy 2001). However, correct interpretation of field isotopic data requires knowledge of both the isotopic fractionation that occurs during the metabolic and physiological processes and the natural variations arising from ecological factors, such as changes in feeding habitats via migrations or prey switching. A better understanding of metabolic and physiological effects on isotopic fractionation under laboratory controlled conditions is critical to validate the use of stable isotopic approaches in natural ecosystem studies. Due to the unique life cycles of marine mammals, controlled feeding trials using captive harbor seals as experimental models are a prerequisite for field studies of this species. So far few laboratory feeding trials under controlled conditions have been conducted, although stable isotopic analysis has been increasingly applied for studies of wild pinnipeds, as mentioned above.

Harbor seals *(Phoca vitulina)* are small pinnipeds that are widely distributed throughout the coastal regions of the Northern Hemisphere. The populations of harbor seals and several other marine mammal species in Alaska have declined significantly over the last two decades and the cause is not known (Pitcher 1990; Frost et al.1994; Small and DeMaster 1995). One hypothesis is that nutritional stress due to food limitation

contributed to these population declines. Studies suggest that a climate regime shift or human activities such as overfishing, may have resulted in decreasing carrying capacity (Beamish 1993; Schell et al.1998) and changes in predominant prey fish species of the Gulf of Alaska and Bering Sea ecosystems (Niebauer and Hollowed 1993, Anderson and Piatt 1999). Specifically, changes in predominant prey fish species in late 1970's from Pacific herring (*Clupea pallasi*) to walleye pollock (*Theragra chalcogramma*) might have caused nutritional stress in harbor seals or other marine mammals, due to the relatively low energy density of walleye pollock compared with that of Pacific herring.

As part of an ongoing investigation of harbor seal health and nutrition, we assessed the relationship between isotopic composition of captive harbor seals and their two fish diets, either exclusively Pacific herring or walleye pollock, during a two-year controlled feeding trial. These two prey fish species have been hypothesized to represent different feeding regimes potentially affecting the survival of wild seals. Carbon and nitrogen isotope ratios in harbor seal serum and RBCs were monitored, to investigate the metabolic effects on isotopic fractionation in response to different prey fish diets and seasonal physiological changes. A conceptual model was proposed to explain the metabolic effects on the varying isotopic fractionation between harbor seals and their diets. In addition, carbon and nitrogen isotope ratios in several tissue types from harbor seals were compared, with the goal of elucidating how the chemical composition of tissue proteins affected tissue-specific isotope ratios.

MATERIALS AND METHODS

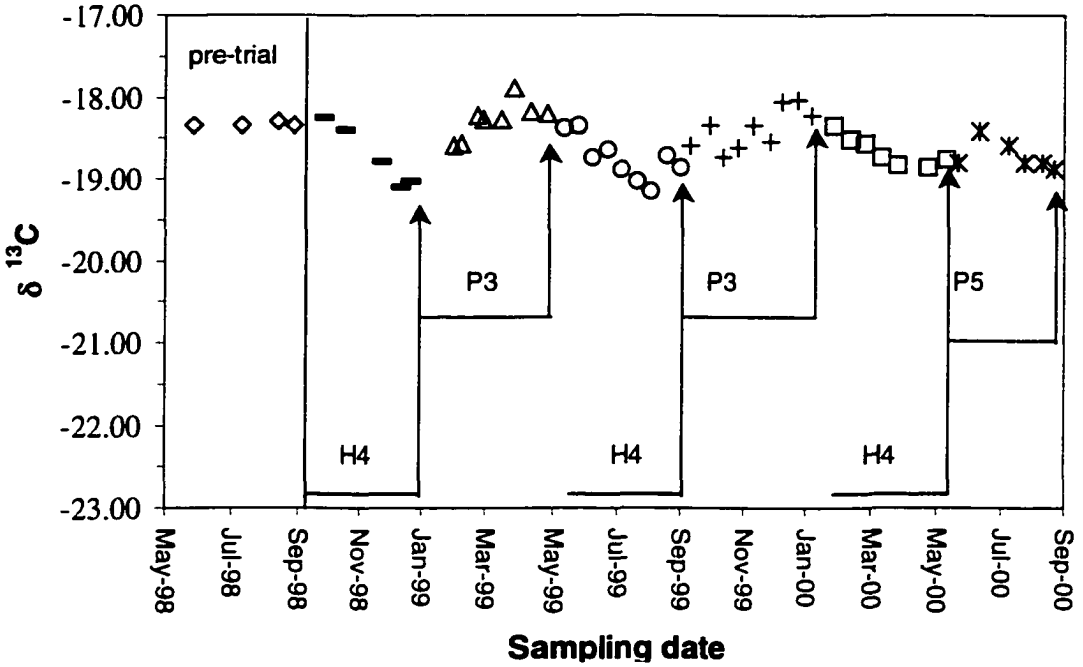
The study of four adult, captive harbor seals was conducted via a 2-year controlled feeding trial, from 15 Sept. 1998 to 15 Sept. 2000, at the Alaska SeaLife Center (ASLC) in Seward, Alaska. Stable isotopic measurements of samples were undertaken at the isotope ratio mass spectrometry facility of University of Alaska Fairbanks. Refer to Controlled Feeding Trial and Harbor Seal Blood Sampling and Stable Isotope Analysis in Chapter 2 for the details of feeding schedule, blood sampling protocols and stable isotopic analytical methods.

RESULTS

$\delta^{13}C$ and $\delta^{15}N$ signatures in serum and RBC proteins of captive harbor seals

Fig. 3.1a, b show the variations of $\delta^{13}C$ and $\delta^{15}N$ in blood serum of harbor seal Travis in response to different fish diets. Natural abundance $\delta^{13}C$ signatures in some feeding cycles are also shown in serum (Fig. 3.2a, b, c) and RBCs (Fig. 3.3a, b, c) of the other three harbor seals. In general, $\delta^{13}C$ values in serum of harbor seals tracked their different fish diets. As shown in Fig. 3.1a and Fig. 3.2a, depleted serum $\delta^{13}C$ values were observed in harbor seal Travis and Pender when they were fed on a herring diet, due to the depleted $\delta^{13}C$ values in herring tissues. After the diet was switched to pollock, the relatively enriched $\delta^{13}C$ values in pollock tissues led to a progressive increase in serum $\delta^{13}C$ values in both Travis and Pender, which then gradually decreased upon switching back to herring and so on. The reverse patterns were observed in harbor seal Poco (Fig.

a)



b)

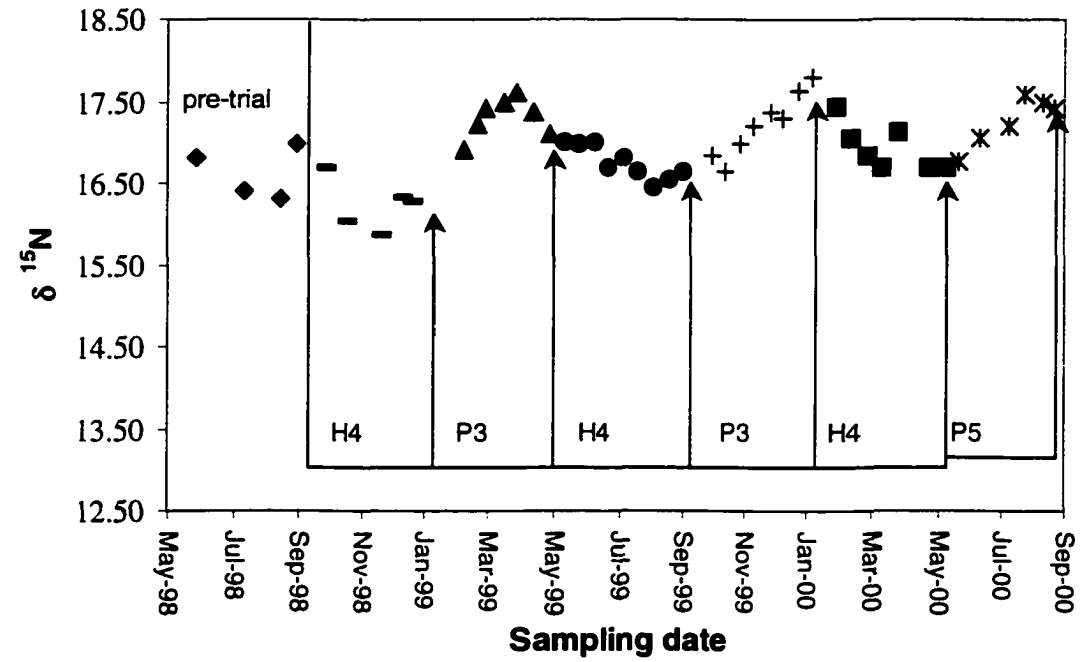


Fig. 3.1 The a) $\delta^{13}C$ and b) $\delta^{15}N$ variations in serum of Travis in response to different fish diets. The dates of diet switching are indicated by vertical lines and the isotopic compositions of the diets are shown as horizontal lines.

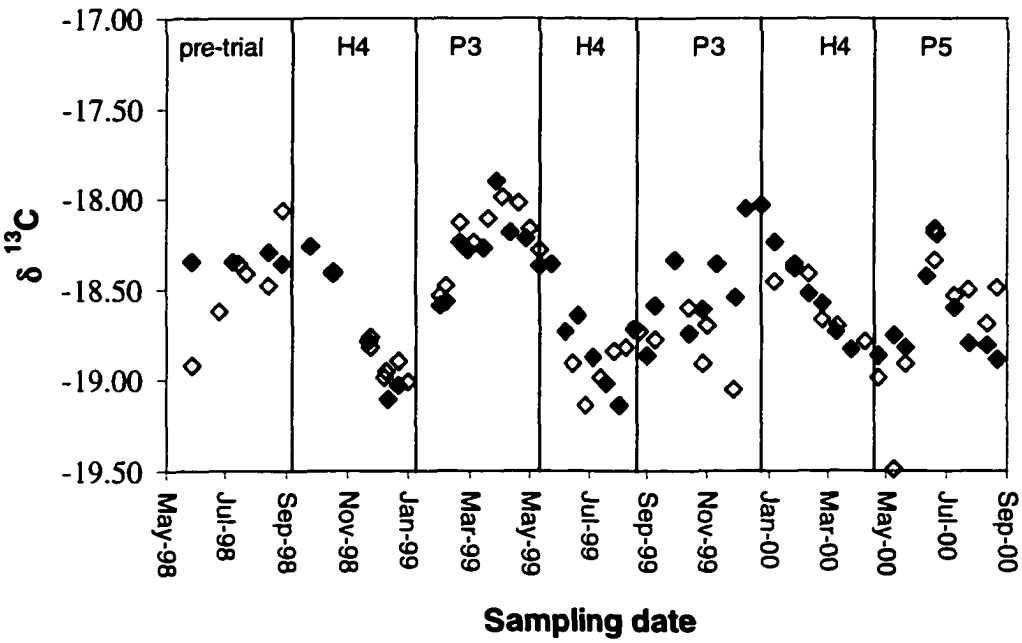


Fig. 3.2a The $\delta^{13}C$ variations in serum protein of the other three captive harbor seals in response to different fish diets. Pender (◇) and Travis (◆) on the same feeding schedule.

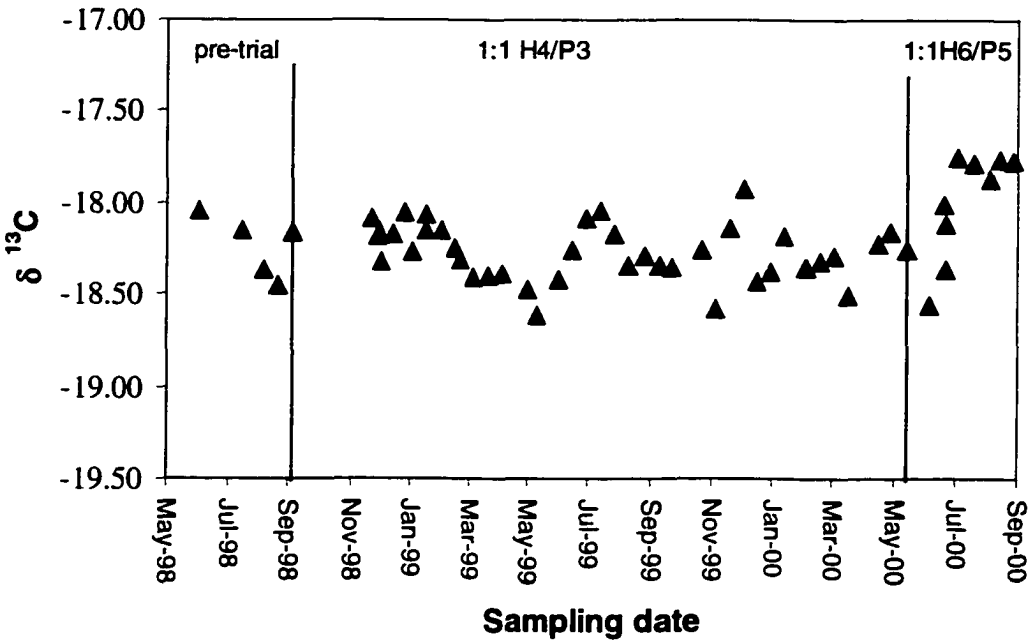


Fig. 3.2b The $\delta^{13}C$ variations in serum protein of the other three captive harbor seals in response to different fish diets. Snapper (▲) on a constant 1:1 mixed diet.

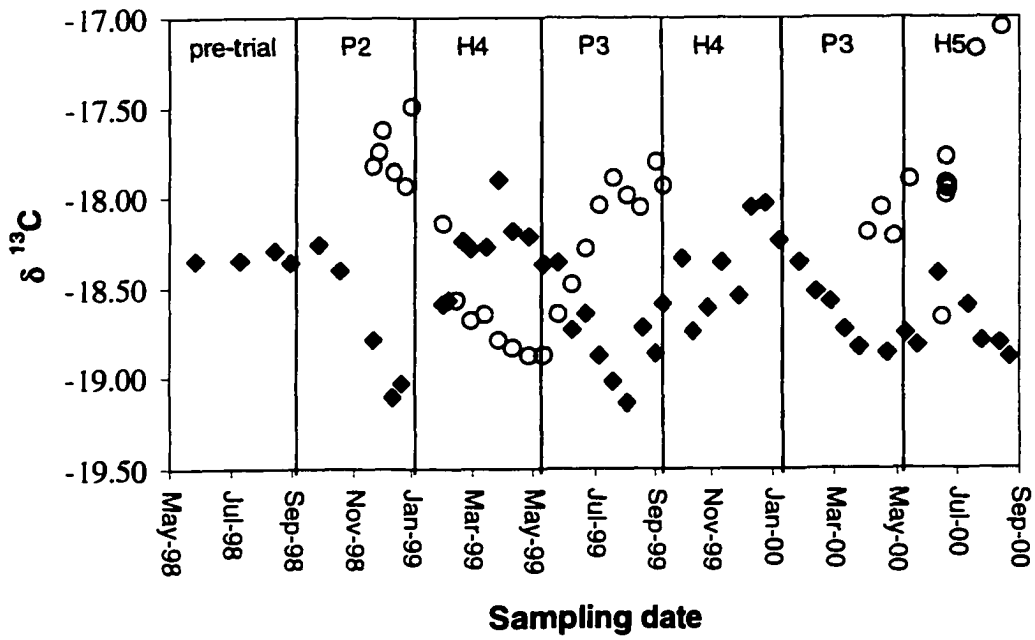


Fig. 3.2c The $\delta^{13}C$ variations in serum protein of the other three captive harbor seals in response to different fish diets. Poco (○) was fed the diet indicated by the labels and Travis (◆) was fed the reverse diet.

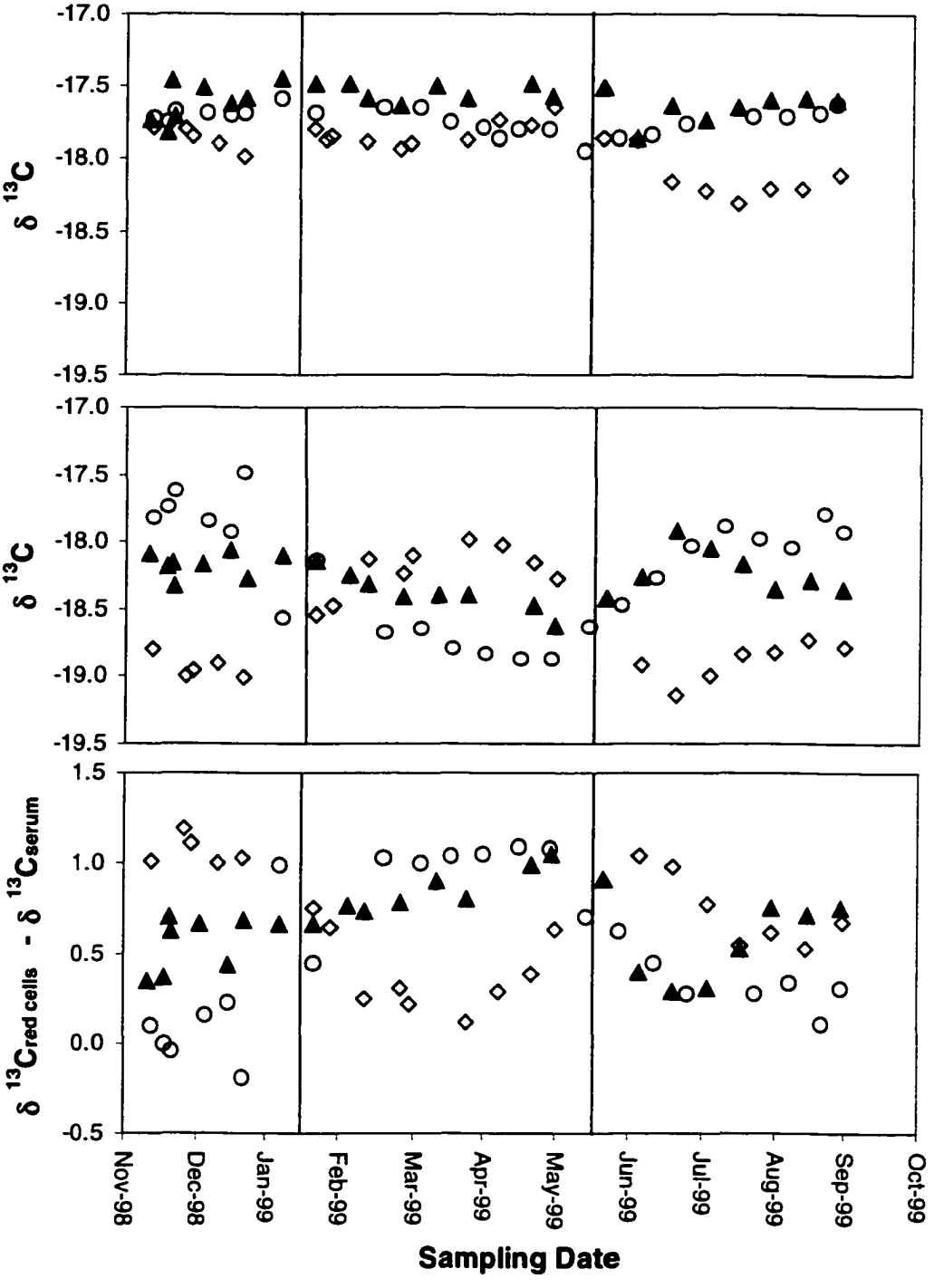


Fig. 3.3 The $\delta^{13}C$ variations in harbor seals Poco (○), Pender(◇) and Snapper (▲) in response to different fish diets. a) $\delta^{13}C$ in RBCs; b) $\delta^{13}C$ in serum and c) differences of $\delta^{13}C$ values between RBCs and serum. Note that the scale of the X-axis differs from Fig. 3.1 and Fig. 3.2.

3.2c), who started with initial diet of pollock and switched to herring. As expected, no such pattern was found in harbor seal Snapper on a 1:1 mixed diet (Fig. 3.2b).

As shown in Fig. 3.1b, serum $\delta^{15}N$ values in harbor seal Travis co-varied with $\delta^{13}C$ signatures as diet switched, although there were no statistically significant differences in fish tissue $\delta^{15}N$ values between herring (Batch 4) and pollock (Batch 3) (t-test, $p < 0.005$). This suggested a more active nitrogen metabolism and increased fractionation when Travis was fed pollock.

The $\delta^{13}C$ values of harbor seal RBCs were indistinguishable for the two fish diets, despite the fact that there was a 2.07‰ difference in carbon isotopic composition between herring (Batch 4) and pollock (Batch 3). As shown in Fig. 3.3a,b, $\delta^{13}C$ values in RBCs exhibited much less variation in response to dietary switches than did those in serum, although the muted patterns of variation were similar. This was expected, as ^{15}N-labeled amino acid tracer experiments showed that the turnover rate of RBCs was much slower than that of serum, with a half-life of approximately 100 days (refer to Chapter 5). Thus RBCs integrate any change in isotopic abundance due to diet shifts over a period of several months. Overall, RBC proteins were more enriched in ^{13}C than those in serum. Paired $\delta^{13}C$ serum and RBC values demonstrated that RBC proteins were enriched in all three captive harbor seals, independent of diet (Fig. 3.3c). Such isotopic differences between serum and RBCs might result from the different amino acid compositions of these two tissue types.

Metabolic effects on carbon and nitrogen isotopic fractionation

Information including catch location/time, feeding periods and the mean ± SE of stable isotope ratios of five batches of diet fish used in the controlled feeding trial are given in Table 3.1. Their carbon and nitrogen isotope ratios are shown in Fig. 3.4. By comparing carbon isotope ratios of harbor seals ($\delta^{13}C$ in serum and RBC proteins) and the corresponding fish diet ($\delta^{13}C$ in whole tissues of herring or pollock), the carbon trophic enrichments were derived for Travis (Fig. 3.5) and the other three seals (Fig. 3.6). As shown in Fig. 3.5 and 3.6, carbon trophic enrichments varied with diets. Harbor seals fed herring fractionated ^{13}C to a greater extent than those fed pollock. The mean carbon trophic enrichment in serum proteins was 4.18‰ on a herring diet (n=62) compared to 2.35‰ on a pollock diet (n=67). The control seal Snapper fed on a constant 1:1 mixed diet had an intermediate value of 3.36‰ (n= 51). Since the above calculations of the trophic enrichments were not based on the same tissue types in consumers and diets, tissue differences and varying lipid contents in tissues may place some limits on these inferences. However, the relative patterns of trophic enrichment in response to different diets should be unaffected, although the absolute values of trophic enrichment might differ.

The mean nitrogen trophic enrichment in serum of harbor seal Travis was 3.83‰ on the herring diet (n=18) and 4.55‰ on the pollock diet (n=22) (Fig. 3.5). This difference in the trophic shift of nitrogen was opposite that of carbon, whose trophic enrichment was more pronounced on the herring diet. The higher nitrogen trophic

Table 3.1 The mean ± SD of carbon and nitrogen isotope ratios, feeding periods, and catch location and time for the five batches of diet fish used in the controlled feeding trial

Batches	Feeding periods	$\delta^{13}C$ (‰) Mean ± SD	$\delta^{15}N$ (‰) Mean ± SD	n	Catch Location and Time
Herring					
Batch 4 (H4)	11/98-05/00	-22.82 ± 0.72	12.76 ± 0.44	72	PWS, Nov. 98
Batch 6 (H6)	06/00-09/00	-21.34 ± 0.36	12.02 ± 0.21	10	Petersburg Dec. 00
Pollock					
Batch 2 (P2)	09/98-02/99	-19.72 ± 0.32	13.96 ± 0.37	15	GOA, Mar. 98
Batch 3 (P3)	02/99-05/00	-20.75 ± 1.14	12.68 ± 0.89	30	GOA, Jan 99
Batch 5 (P5)	06/00-09/00	-21.13 ± 0.41	12.86 ± 0.52	3	Unknown

Diet pairs for Snapper with a 50% herring and 50% Pollock mixed diet

H4—P2	11/98-02/99
H4—P3	02/99-05/00
H6—P5	06/00-09/00

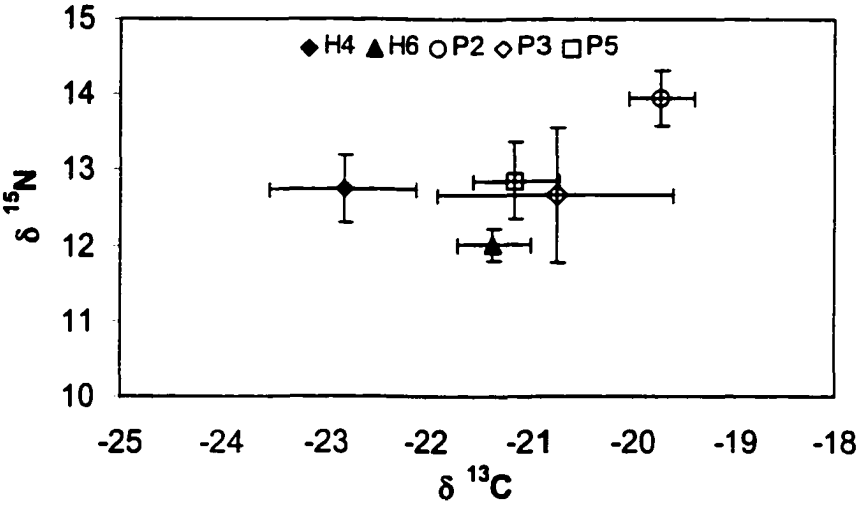


Fig. 3.4 The mean ± SD of the $\delta^{13}C$ and $\delta^{15}N$ values in the five batches of fish diets used in the controlled feeding trial. H4 (◆n=72); H6 (▲ n= 10); P2 (○ n= 15); P3 (◇ n=30); P5 (□ n=3).

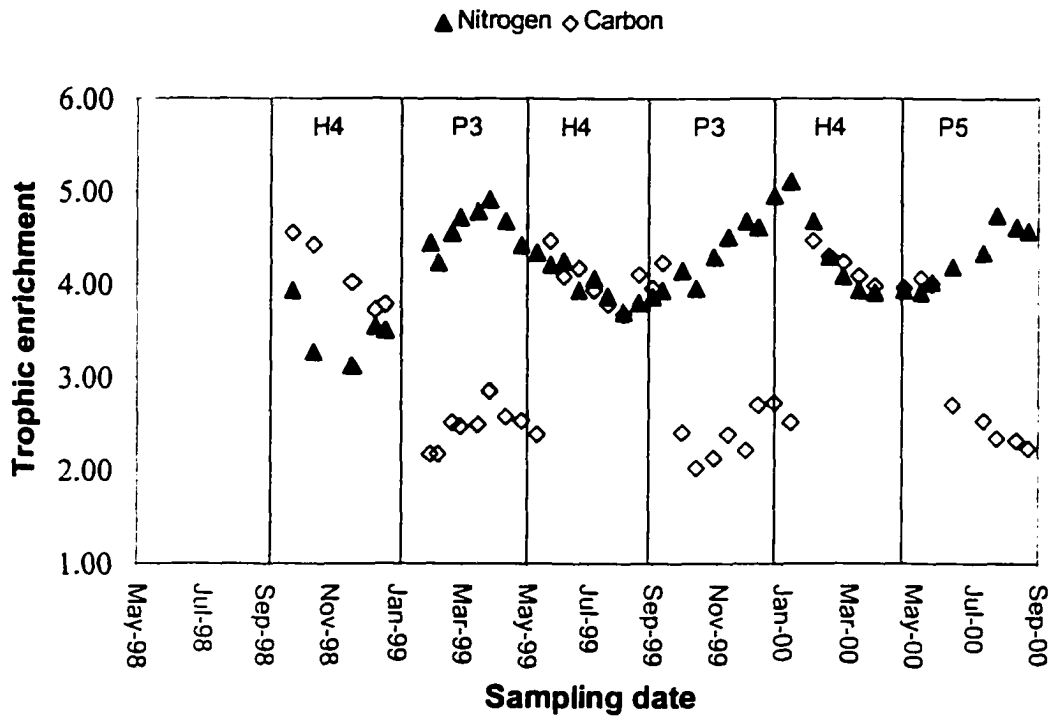


Fig. 3.5 Trophic enrichment of carbon (◇) and nitrogen (▲) in harbor seal Travis in response to different fish diets.

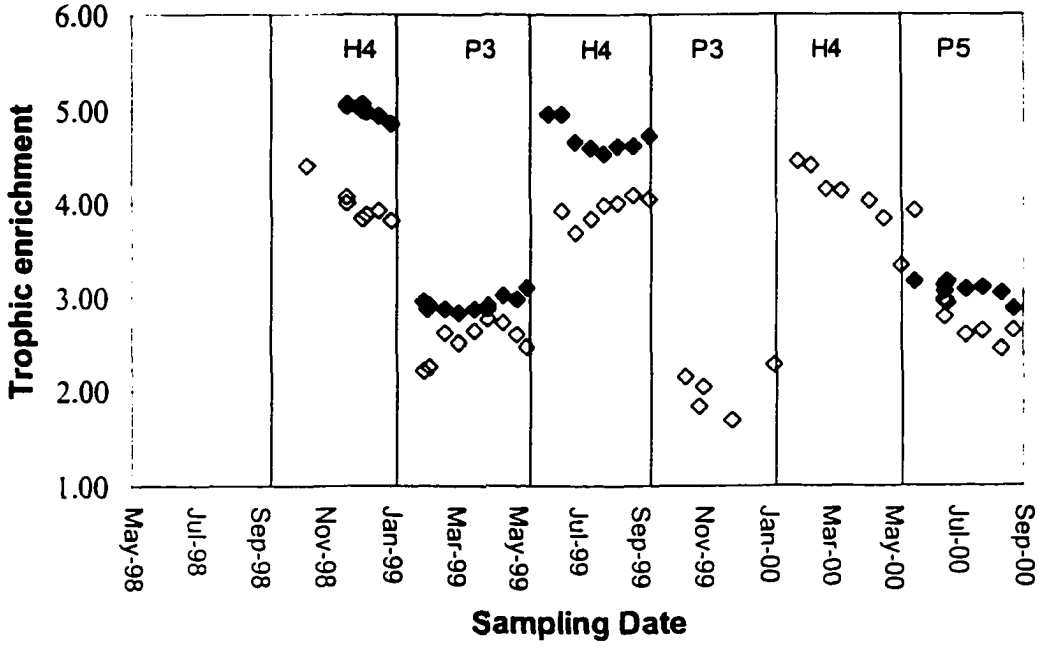


Fig. 3.6a Carbon trophic enrichments in harbor seal Pender in response to different fish diets. ◇ serum; ◆ RBCs.

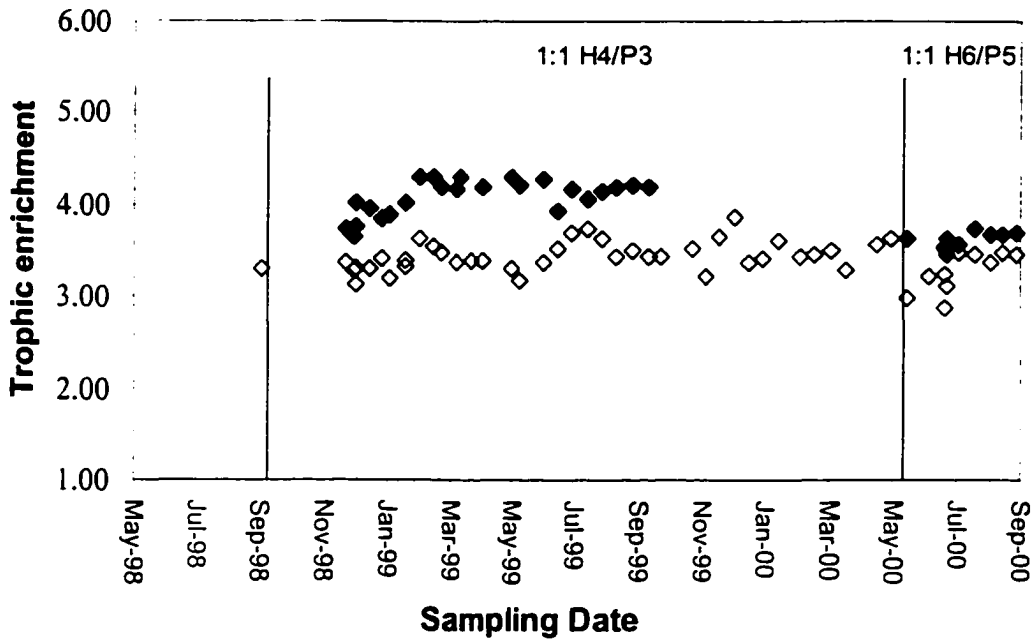


Fig. 3.6b Carbon trophic enrichments in harbor seal Snapper in response to different fish diets. ◇ serum; ◆ RBCs.

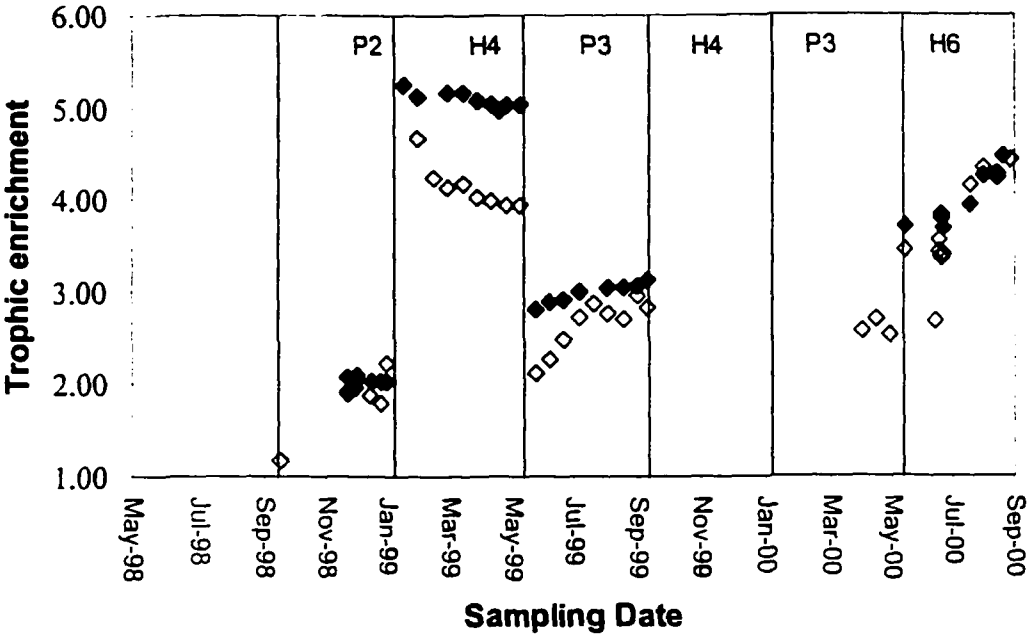


Fig. 3.6c Carbon trophic enrichments in harbor seal Poco in response to different fish diets. ◇ serum; ◆ RBCs.

enrichment in Travis might be the result of a coincident higher protein intake on the pollock diet. As shown in Fig. 3.7, the daily body protein intake of Travis was considerably higher when pollock was fed.

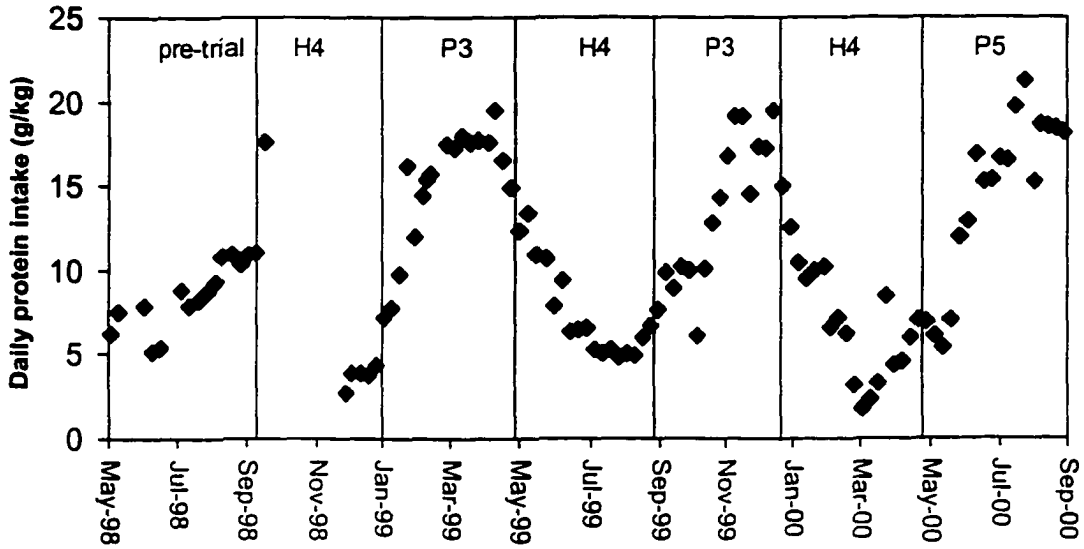
Dietary turnover

Taking serum $\delta^{13}C$ variations in Travis as representative of responses to diet switches (Fig. 3.1a), serum $\delta^{13}C$ values reflected the carbon isotopic signature of the new fish diet immediately following the diet switching, and increasingly resembled that of the new diet over time. However, no plateau in isotopic composition was reached even after 120 days (one feeding cycle of one consistent diet), possibly due to the relatively large body mass of the seal. The changes in isotopic composition of serum after the diet switch were basically attributable to two processes: metabolic tissue replacement and growth. As shown in Fig.3.8, the estimated isotopic turnover rates were not uniform for every feeding cycle. Although metabolic tissue replacement was probably the major component of turnover for marine mammals (Hobson and Clark 1992), growth might also have contributed, as demonstrated by the variations of body mass of Travis in different feeding cycles (Fig. 3.9). However, the difference in carbon isotopic values of the two fish diets was small and prevented an exact estimation of isotopic turnover rates.

$\delta^{13}C$ and $\delta^{15}N$ values in fur, nails, whiskers, serum and RBCs

Fig. 3.10a, b, c show the carbon and nitrogen isotope ratios in different tissues from the three captive harbor seals. The most enriched $\delta^{15}N$ values were found in serum,

a)



b)

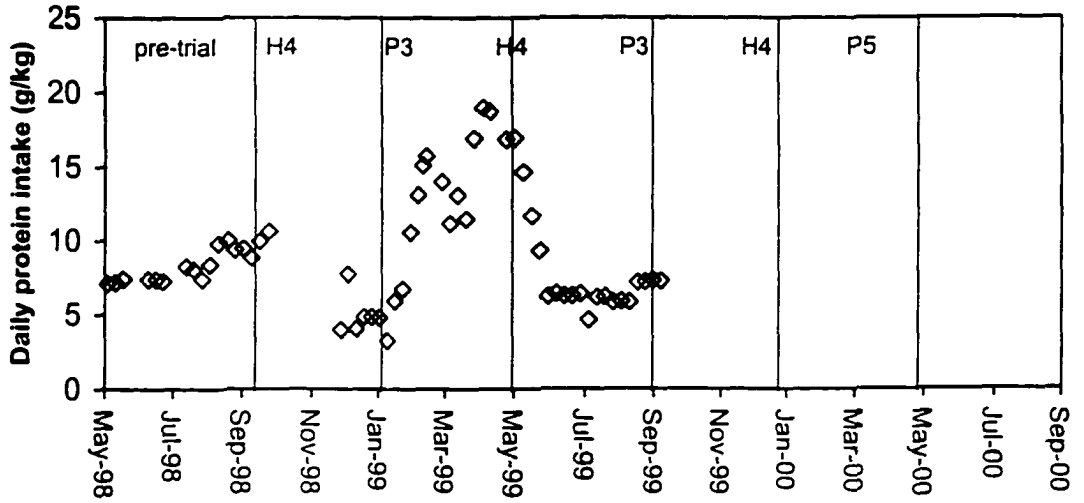


Fig. 3.7 Variations of daily protein intake (g protein per kg body mass per day) of a) Travis (◆) and b) Pender (◇) in response to different fish diets.

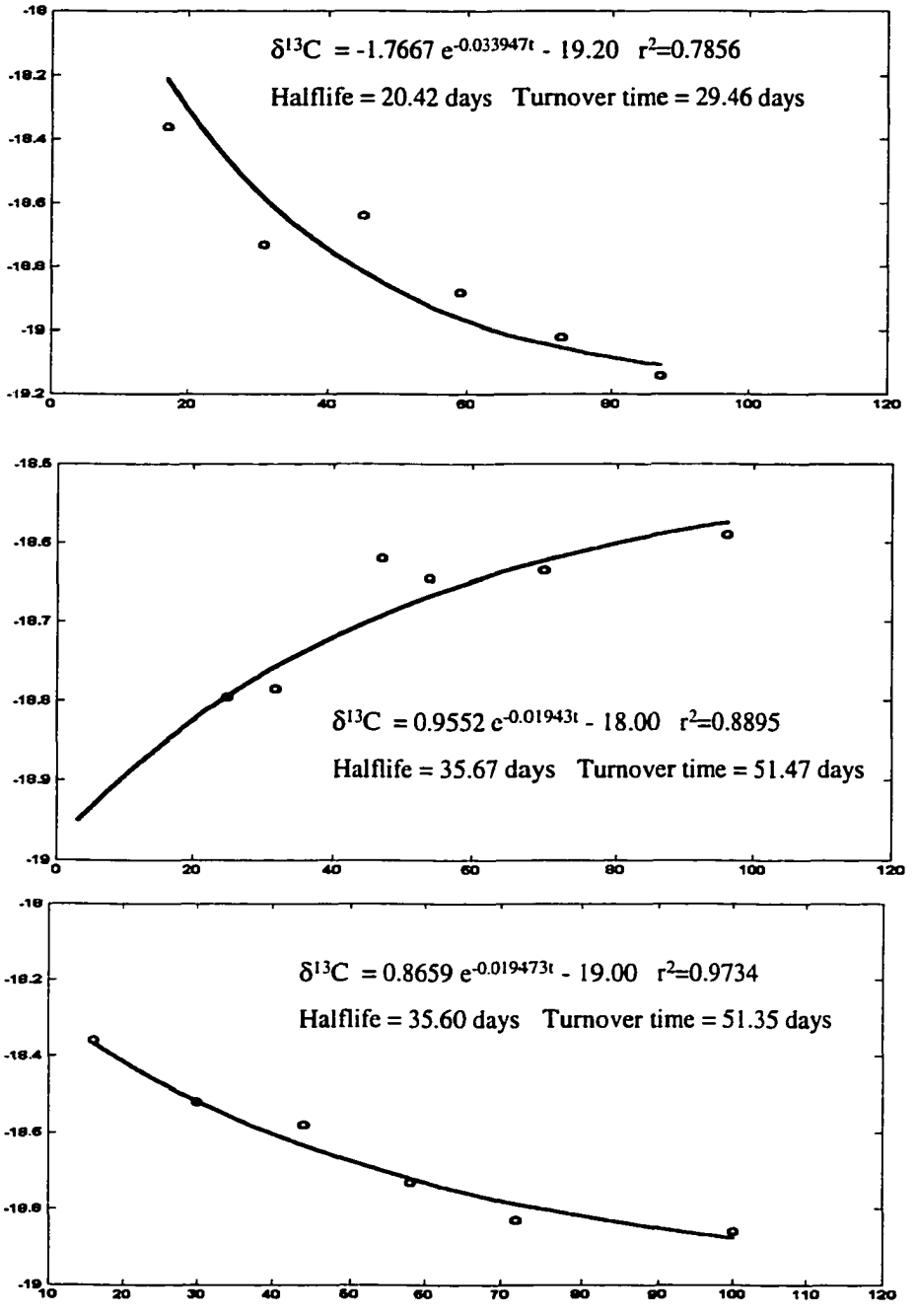


Fig. 3.8a Dietary $\delta^{13}C$ turnover curves of three feeding cycles in Travis. Top and bottom: the diet was switched from pollock to herring; middle: the diet was switched from herring to pollock.

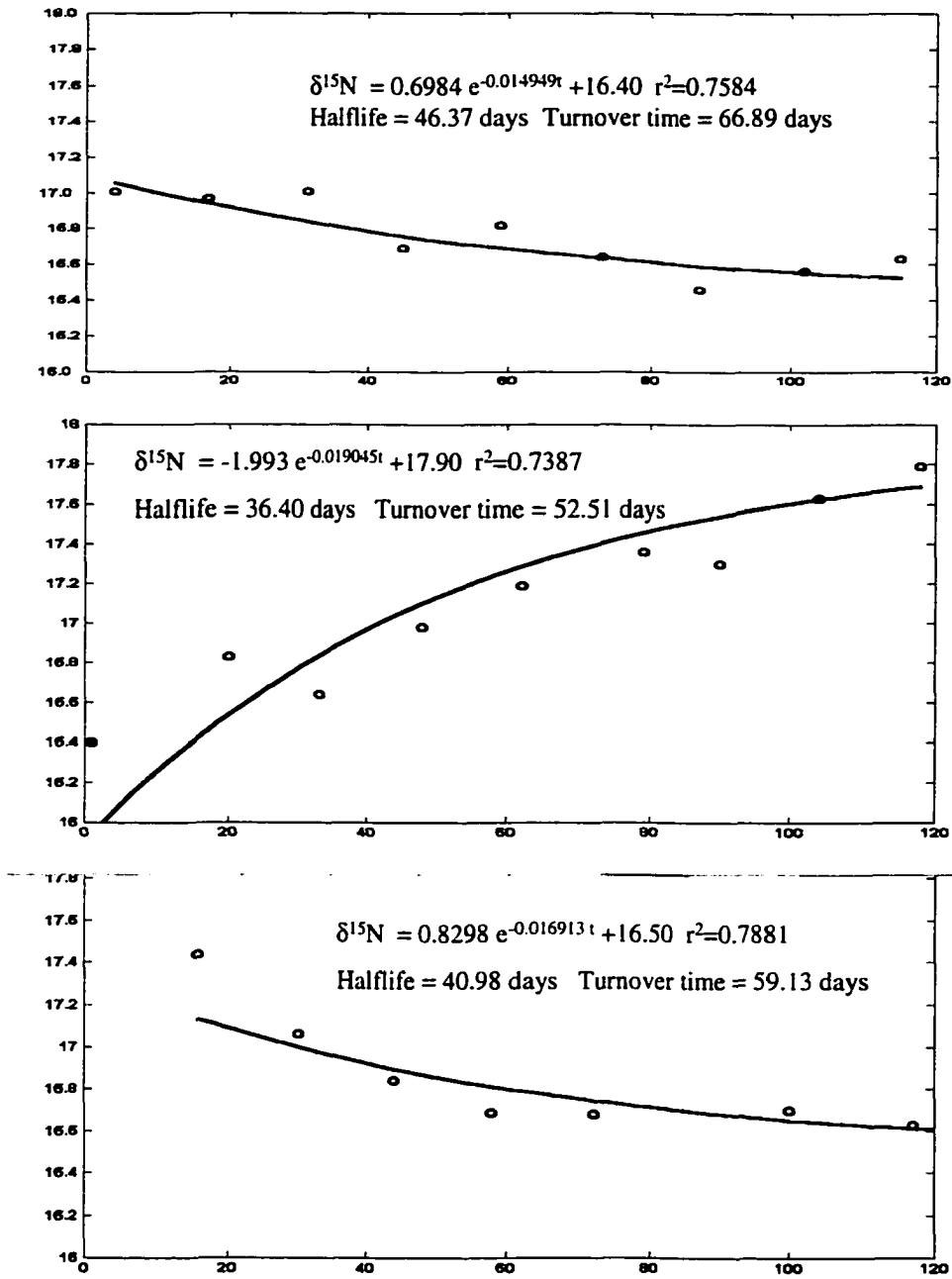


Fig. 3.8b Dietary $\delta^{15}N$ turnover curves of three feeding cycles in Travis. Top and bottom: the diet was switched from pollock to herring; middle: the diet was switched from herring to pollock.

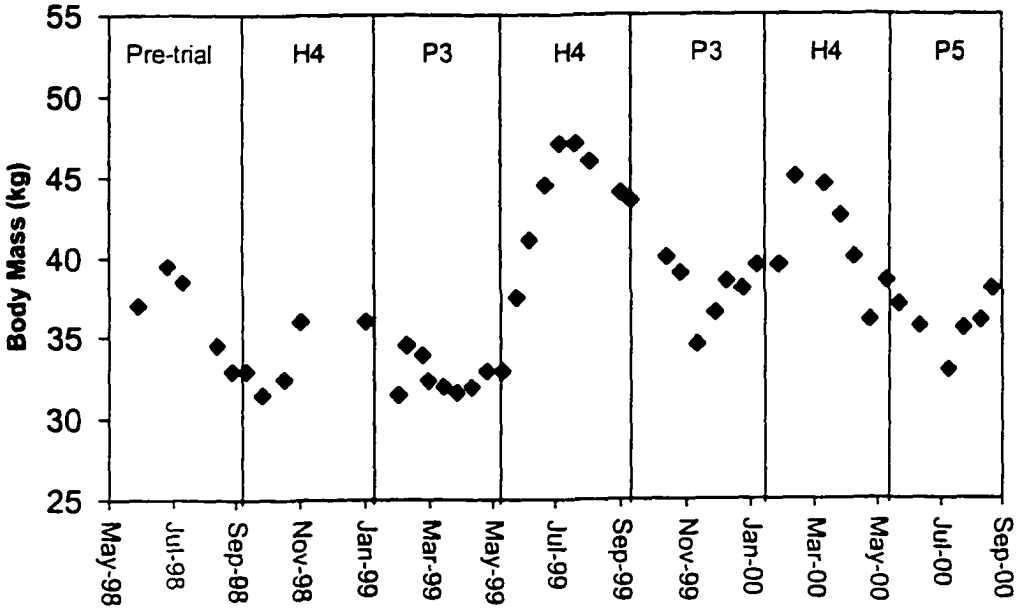


Fig. 3.9 Variations of the body mass of harbor seal Travis over the course of the controlled feeding trial.

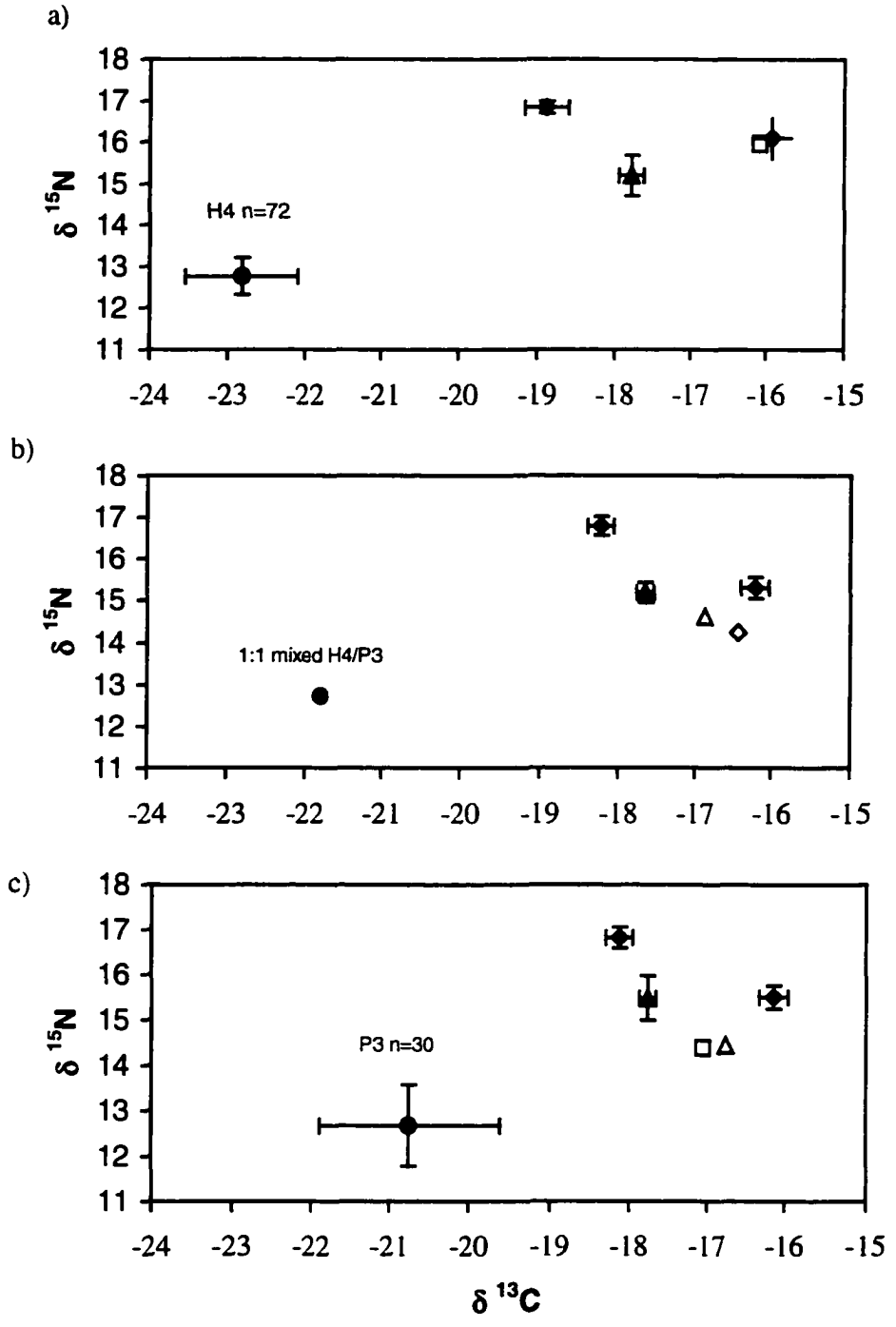


Fig. 3.10 The carbon and nitrogen isotopic compositions in different tissues of the three captive harbor seals.a) Pender on a herring diet; b) Snapper on a 1:1 mixed diet and c) Poco on a pollock diet. ● diet; ○ serum; ▲ RBC; □ nail; △ fur and ◆ whisker. Fur and nails represent the mean of replicates.

followed by RBCs or whiskers, and the most depleted $\delta^{15}N$ values were in fur or nail, both keratinous proteins. In contrast, $\delta^{13}C$ values were in the order of whisker > fur or nail > RBCs > serum. No effects on isotopic composition were noted due to either molting or breeding activities.

DISCUSSION

Carbon isotopic composition and its trophic isotopic fractionation

Generally speaking, carbon isotope ratios are more conservative than those of nitrogen when they are transferred up the trophic ladder. The relatively small trophic enrichments (0 -1 ‰) between consumers and their diets allow naturally occurring carbon isotope ratios to be important tracers in distinguishing relative contributions of two distinct carbon sources in ecosystems. e.g., freshwater vs. marine, inshore vs. offshore, pelagic vs. benthic, etc. (Fry and Sherr 1984; Peterson et al. 1985; Dunton et al. 1989; Hobson et al. 1993; Fantle et al. 1999). A combination of carbon and nitrogen isotope data has proven to be useful, because co-variances of $\delta^{15}N$ with $\delta^{13}C$ may serve as good indicators of geographical migration or prey switching (Schell et al. 1989; Saupe et al. 1989; Schell et al. 1998).

Results from this study demonstrate that carbon isotopic fractionation varies in response to different diets, suggesting that diets have important influences on the observed isotopic composition in wild harbor seals. An average 2.07‰ difference in $\delta^{13}C$ values between herring (-22.82‰, n = 72) and pollock (-20.75‰, n = 30) produced less

than 1‰ difference in serum $\delta^{13}C$ signatures of seals, due to the greater carbon trophic enrichment for herring (4.18‰) than for pollock (2.35‰). In fact, dietary effects on trophic enrichment are not uncommon and have been documented in several controlled laboratory studies (DeNiro and Epstein 1978; 1981; Mizutani et al. 1991; Hobson and Clark 1992a, b; Hobson and Clark 1993). Trophic enrichments vary depending on the consumer species or the prey species consumed. Differences in lipid contents between herring and pollock may account for partial differing carbon trophic enrichments in this study, since $\delta^{13}C$ values in lipids are generally depleted by 6‰, compared with those in proteins (DeNiro and Epstein 1978; Tieszen et al. 1983). A recent study by Focken and Becker (1998) showed a threefold difference in carbon trophic enrichment in two fish species during a controlled feeding trial, for both whole and defatted tissues.

Nitrogen isotopic fractionation between harbor seals and their diets

Nitrogen isotope ratios reflect both the trophic level of consumers and their diet. A stepwise nitrogen isotopic fractionation (~3.2‰) between consumers and their diets (the so-called trophic enrichment or trophic shift) has been observed from plants to herbivores to carnivores in both terrestrial and marine ecosystems and in both field and laboratory studies (DeNiro and Epstein 1981a,b; Minagawa and Wada 1984; Schoeninger and DeNiro1984; Sealy et al. 1987; Owens 1987; Peterson and Fry 1987; Rau et al. 1992; Hobson and Welch 1992; reviewed by Michener and Schell 1994).

In contrast to the lesser carbon trophic enrichment between harbor seals and pollock compared with herring, the reverse pattern for nitrogen trophic enrichment was observed. Unfortunately, there were no data available for RBCs due to the concurrent ^{15}N-enriched amino acid tracer experiments. Harbor seals fed pollock fractionated nitrogen to a greater extent (4.55‰, n=22) and tended to have higher $\delta^{15}N$ values in their serum proteins than those fed on herring (3.83‰, n=18). Although the 0.72‰ difference in nitrogen trophic enrichment is smaller than the 1.83‰ difference in carbon, the dietary effect is still significant as demonstrated by Fig. 3.1b and 3.5. The $\delta^{15}N$ values increased progressively as the diet was switched from herring to pollock, although there were no statistically significant differences in $\delta^{15}N$ values between the herring and pollock (t-test, $p<0.001$).

This diet dependent trophic enrichment can be attributed to differing metabolic isotopic effects in response to these two diet regimes. As Fig. 3.7 shows, the daily body protein intake of harbor seal Travis differs considerably for the herring and pollock diets. The daily body protein intake of Travis is greater on the pollock diet and smaller on herring. Proximate analysis reveals that herring has more than twofold higher lipid, but roughly one-third lower protein, than pollock based upon dry weight (Castellini et al. 2002). Our amino acid composition data also demonstrate that the total hydrolysable amino acid concentrations (μmol/g dry weight of whole fish tissue) are on average 1.5 times higher in pollock than in herring (see Chapter 6 for details). However, the differences in daily body protein intakes between these two fish diets were not only the

result of protein content of the fishes, but partly due to the increased total amount of pollock eaten.

We hypothesize that changes in protein metabolic pathways, including protein synthesis, breakdown and urea excretion, in response to different dietary protein intake, are responsible for the varying nitrogen isotopic fractionation in harbor seal serum proteins. Since isotopic fractionation mainly occurs in transamination or deamination reactions during protein catabolism (Gaebler et al. 1966; Macko et al. 1987), greater isotopic fractionation may occur on the pollock diet, as the rates of both amino acid breakdown and urea excretion can be stimulated by high dietary protein intake. The effect of protein content in human diet on urea excretion rates is well known (Epstein et al. 1957). A high rate of excretion of ^{14}N as urea results in greater nitrogen isotopic fractionation between consumers' tissue and their diet (reviewed by Ambrose 1991). As a result, nitrogen isotope ratios in consumers' tissues are more enriched in $\delta^{15}N$ on a high protein diet than on a low protein diet (Sick et al. 1997). The magnitude of fractionation may also reflect the physiological and nutritional conditions of the consumer, due to the regulation of protein metabolic pathways based on the consumer's endogenous requirements.

Varying nitrogen isotopic fractionations have been documented in other studies. Hobson and Clark (1992b, 1993) suggested that significantly enriched tissue $\delta^{15}N$ values of birds were due to fasting or nutritional stress when birds were raised in laboratory with

restricted food intake or when wild birds were sampled during their natural fasting period. Enriched $\delta^{15}N$ values in starving animals are caused by the increased loss of isotopically lighter ^{14}N due to metabolism of protein from their own muscles instead of dietary proteins. Thus, the animals become progressively more enriched in ^{15}N over the course of starvation (Gannes et al. 1997). Fantle and coworkers (1999) observed that a slowly growing juvenile blue crab that fed on a protein-poor detritus diet fractionated nitrogen much more than a rapidly growing crab fed on a high protein zooplankton diet. The larger nitrogen fractionation occurred on the protein-poor detritus because the limited supply of basic metabolic energy resulted in the breakdown of the crabs' own proteins and lipids to meet their energy requirements. However, both herring and pollock contain high protein and energy density. Hence, it is unlikely that malnutrition play any role in the results of this study.

Comparison of tissue-specific isotope ratios

It is known that different tissues in an organism vary in their isotopic composition (DeNiro and Epstein 1981; Tieszen et al. 1983; Minagawa and Wada 1984; Hobson et al. 1996). Several studies have documented the general relationships among $\delta^{13}C$ values with hair > muscle > liver > fat (Tieszen et al. 1983; Hobson and Clark 1992a,b; Hobson and Clark 1993; Hobson et al. 1997). Our data for captive harbor seals yielded an order of $\delta^{13}C$ values of whisker = nail > fur > RBCs > serum and an order of $\delta^{15}N$ values of nail = fur < whisker = RBCs < serum. The order was consistent in all three seals despite their differing diets. $\delta^{13}C$ values in RBCs were always enriched compared with serum proteins,

independent of diet (Fig. 3.3c and Fig. 3.10). Hobson et al. (1997) found that blood plasma $\delta^{13}C$ values were depleted by 1.8 ± 0.3 ‰ in bears compared with the cellular fractions. In contrast, $\delta^{15}N$ values in RBCs are generally lower than those in serum proteins (Fig. 3.10). These tissue-dependent isotope ratios may be closely associated to tissue-specific amino acid and fatty acid compositions and the isotopic compositions in these individual components. For example, since glycine residues comprise as much as 33% of the total amino acid residues in bone collagen, the greater $\delta^{13}C$ values of glycine compared to those of other amino acids (refer to Chapter 4) may explain the observation that carbon isotope ratios in bone collagen proteins are always more enriched than in other tissues from the same organism (Hare et al. 1991; Hobson and Clark 1992a).

CONCULUSION

The controlled feeding of captive harbor seals provided us a unique experimental model for better understanding of metabolic and physiological effects on isotopic fractionation, by excluding ecological factors. This allowed us to test the assumption of constant isotopic fractionation at each trophic level underlying currently accepted food web isotope applications. In general, both $\delta^{13}C$ and $\delta^{15}N$ signatures in harbor seal serum reflected the diets of either herring or pollock, but isotopic fractionation varied with diet. It is hypothesized that changes in the metabolic pathways involved in protein synthesis and urea excretion, in response to differing protein intake, may be responsible for these variable isotopic fractionations, although lipid metabolism may also account for some of

the carbon isotopic effects. Variable and diet dependent isotopic fractionation will complicate interpretation of isotopic data in field studies attempting to use stable isotopic techniques for marine mammal diet reconstruction.

Chapter 4

Natural Abundance Carbon and Nitrogen Isotope Ratios of Individual Amino Acids in Phocids, Prey Fish, and Plankton

ABSTRACT

Reversed-phase HPLC separation and subsequent IRMS measurement was used to determine carbon and nitrogen isotope ratios in individual amino acids of marine organisms, including several phocids. Natural abundance amino acid carbon and nitrogen isotope ratios were measured in protein hydrolyzates of captive harbor seals (*Phoca vitulina*), their fish diets of Pacific herring (*Clupea pallasi*) and walleye pollock (*Theragra chalcogramma*), wild harbor seals and plankton from Prince William Sound and the adjacent Gulf of Alaska, harbor seals from North Atlantic coastal waters, and three additional phocid species, Weddell seals (*Lepthonychotes weddellii*), Ross seals (*Omatophoca rossii*) and crabeater seals (*Lobodon carcinophagus*), from the Antarctic ocean. Isotopic compositions of individual amino acids varied widely in the marine organisms analyzed. Amino acid $\delta^{13}C$ ranged from –33.09‰ to –8.80‰, with the highest values in serine and glycine and the lowest in leucine. Amino acid $\delta^{15}N$ spanned an even greater range, from –7.74‰ to 27.83‰, with the highest values in proline and valine and the lowest in threonine. Amino acids varied more in their carbon and nitrogen isotopic composition within an organism than across trophic levels. This wide range of isotopic composition reflects the distinct biosynthetic pathways for different amino acids. As observed in the bulk tissues, trophic enrichments were evident in both amino acid carbon

and nitrogen isotope ratios between captive harbor seals and their fish diets. The strikingly similar patterns in relative amino acid carbon isotopic composition along the trophic ladder indicate the inherently conservative transfer of amino acid isotope ratios from primary producers to top predators. Differences in amino acid $\delta^{13}C$ values between seals from the north Pacific or Atlantic and their counterparts from the Antarctic reflected the geographic variation of isotope ratios in primary producers. As generally observed for bulk tissues, the magnitude of amino acid $\delta^{15}N$ enrichments along the trophic ladder was generally larger than that of amino acid $\delta^{13}C$, indicating that the metabolic isotopic effects on amino acids were more pronounced for nitrogen. Further, the $\delta^{15}N$ trophic enrichment for different amino acids was not uniform, depending upon the extent to which the given amino acid is involved in transamination or oxidative deamination. Lysine, histidine and phenylalanine usually showed smaller variations in $\delta^{15}N$ values than non-essential and branched-chain amino acids, indicating their more conservative transfer along the food webs. These essential amino acids may thus act as natural "biomarkers" to provide more detailed information on the food sources or habitat usage in studies of foraging ecology.

Key words: $\delta^{13}C$; $\delta^{15}N$; amino acid; isotopic fractionation; phocids; marine organisms.

INTRODUCTION

Compound-specific isotope analyses (CSIA) have received increasing attention in recent years (Macko et al. 1987; Hare et al. 1991; Merritt and Hayes 1994; Fogel et al.

1997; Metges and Petzke 1997; Petzke et al. 1997; Metges and Daenzer 2000; Keil and Fogel 2001). The pioneering work by Abelson and Hoering (1961) revealed that there are substantial differences in the fractionation of carbon isotopes of individual amino acids during the process of photosynthesis. Varying nitrogen isotopic fractionation has been observed *in vitro* in microorganisms, due to the different metabolic pathways involved in transamination reactions during amino acid biosynthesis (Macko et al. 1987). Studies on bone collagen demonstrated comparable, although measurably different, isotopic compositions for modern collagen and fossil bones of several species, despite diverse feeding habits (Hare et al. 1991; Fogel et al. 1997). The identical patterns in relative amino acid isotopic compositions among these different species indicated that amino acids are derived from similar biochemical pathways and sources, regardless of species (Hare et al. 1991).

Compared with conventional isotopic analysis of bulk tissues, compound specific isotopic analysis may provide more fundamental insight into the processes responsible for observed isotopic variations (O'Connell and Hedges 2001). Variations in carbon and nitrogen isotopic compositions of individual amino acids may permit more detailed inferences on food sources or habitat usage by providing insight into metabolic isotopic effects that occur during transfer through a food web. Varying trophic enrichments for different amino acids may indicate the differences in protein assimilation and metabolic pathways between essential and non-essential amino acids (Gaebler et al. 1966). Since essential amino acids come exclusively from dietary proteins, if there is little or no

transamination during their metabolism, these essential amino acids can be used as conservative "biomarkers" to trace the sources of food or habitat usage of consumers. However, few data on individual amino acid isotopic analyses have been published, due to the technical difficulties in isolating pure amino acids and collecting sufficient amounts of nitrogen and carbon for isotope ratio measurement (Petzke et al. 1997; Meier-Augenstein 1999). Using improved techniques, we have obtained individual amino acid carbon and nitrogen isotopic compositions in a variety of marine species, from different trophic levels and geographic locations.

MATERIALS AND METHODS

Blood samples from three captive harbor seals were taken after an overnight fast, following approximately 100 days of feeding one constant fish diet, either Pacific herring (Batch 4) or walleye pollock (Batch 3) or 1:1 mixed herring and pollock diet. Herring Batch 4 and pollock Batch 3 were caught, respectively, from Prince William Sound in November 1998 and the Gulf of Alaska in January 1999. Phytoplankton and plankton samples were collected in the Gulf of Alaska at the shelf break south of Seward, Alaska, using vertically towed plankton nets (mesh size: 35 μm and 183 μm) during June 2001.

Blood samples from wild harbor seals were collected in Prince William Sound in 1994 and archived by freezing at –80° C until analyzed. Blood samples from Atlantic harbor seals were collected during a March 2000 field study located in coastal waters of Maine. Blood samples from Weddell, Ross and crabeater seals were collected in the

Eastern Ross Sea during a December 1999 through January 2000 field study in Antarctica. In all cases, serum and RBCs were separated immediately after the blood collection and frozen at –80° C. Care was taken to avoid contamination of the serum with leukocytes.

All samples were freeze-dried and ground for homogeneity for bulk carbon and nitrogen isotope measurement using a Finnigan Delta S isotope ratio mass spectrometer (Finnigan MAT, Germany). Approximately 20 μg of each freeze-dried sample were hydrolyzed and the amino acids were isolated using a semi-preparative reversed-phase HPLC column, without derivatization. The individual amino acid peaks were collected and weighed in a tin cup for subsequent IRMS measurements. Refer to Stable Isotope Analysis of Individual Amino Acids in Chapter 2 for details of the separation of amino acids and stable isotope analysis of individual amino acids.

RESULTS

Natural abundance carbon and nitrogen isotope ratios in individual amino acids were measured in captive harbor seal serum and RBC proteins, muscle proteins of Pacific herring and walleye pollock, as well as proteins of zooplankton and phytoplankton from Prince William Sound and the Gulf of Alaska. Natural abundance amino acid carbon and nitrogen isotopic compositions were also compared among four wild phocid species, including harbor seals from Prince William Sound and the North Atlantic Ocean and Weddell, Ross and crabeater seals from the Antarctic Ocean. Fig. 4.1 shows the

relationship of amino acid carbon and nitrogen isotopic compositions between captive harbor seal serum proteins and their fish diet. Fig. 4.2 compares the amino acid carbon and nitrogen isotopic compositions between captive harbor seal serum proteins and RBC proteins. Amino acid carbon and nitrogen isotopic compositions in organisms from different trophic levels and in several wild seal species are illustrated in Fig. 4.3 and Fig. 4.4, respectively. Fig.4.5 compares the amino acid carbon and nitrogen isotopic compositions from this study with those from the literature.

DISCUSSION

General patterns in AA isotopic composition

Similar patterns in natural abundance amino acid carbon and nitrogen isotopic compositions are observed in captive harbor seals and their two fish diets (Fig. 4.1), in serum and RBC proteins of captive harbor seals (Fig. 4.2), in organisms from different trophic levels of the North Pacific ecosystem (Fig.4.3) and in Antarctic seals (Fig. 4.4). Amino acids differ more in their carbon and nitrogen isotopic composition within an organism than does any single amino acid across trophic levels, reflecting the distinct biosynthetic pathways for different protein amino acids. Amino acid $\delta^{13}C$ values ranged from –33.09‰ to –8.80‰ with the highest ratios in serine and glycine and the lowest ratio in leucine.

Amino acid $\delta^{15}N$ values spanned an even greater range, from –7.74‰ to 27.83‰, with the highest ratios in proline and valine and the lowest ratio in threonine. Only a few

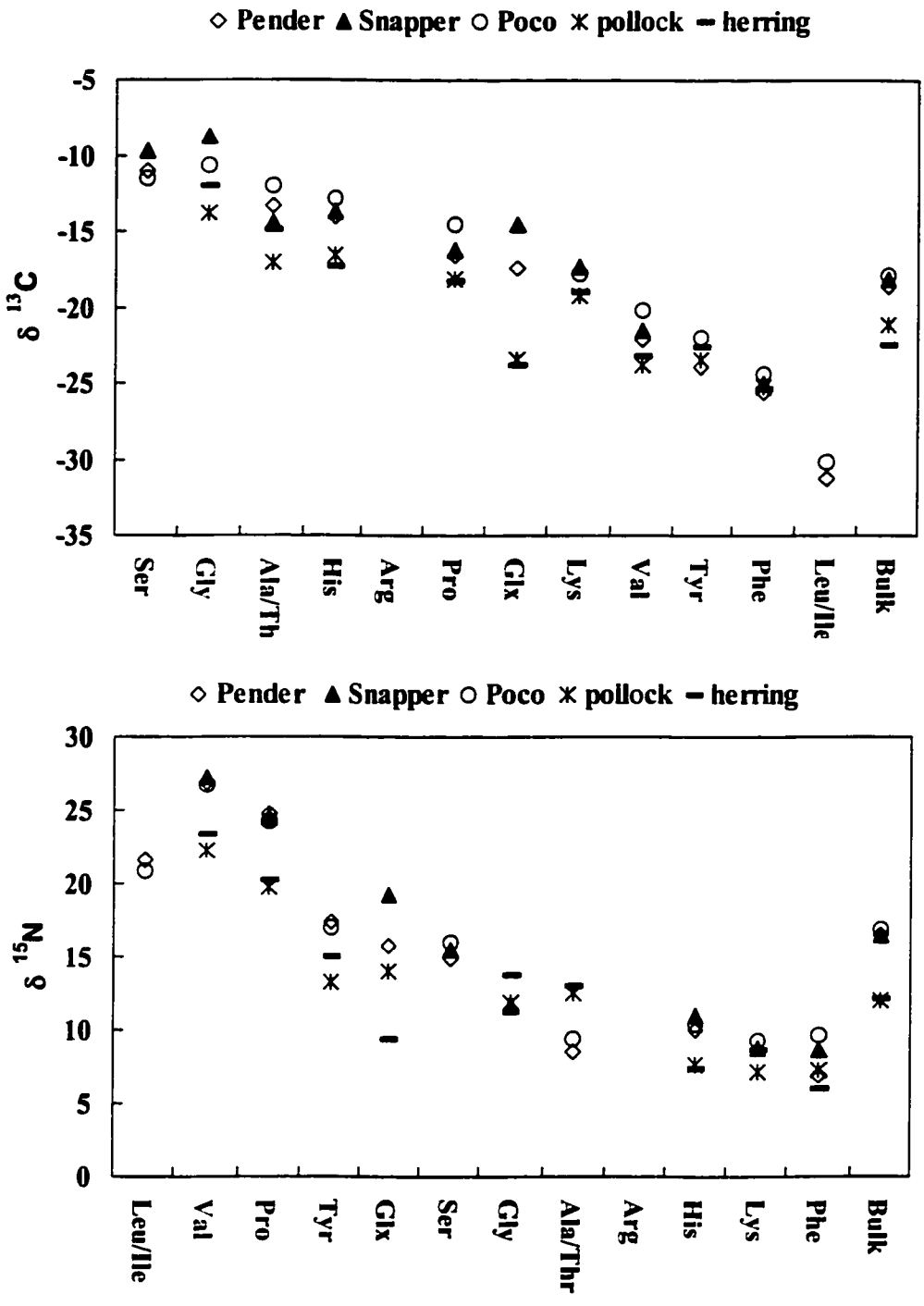


Fig. 4.1 Comparison of the a) carbon and b) nitrogen isotopic compositions of individual amino acids between captive harbor seals and their diets. Each point is a mean of two measurements.

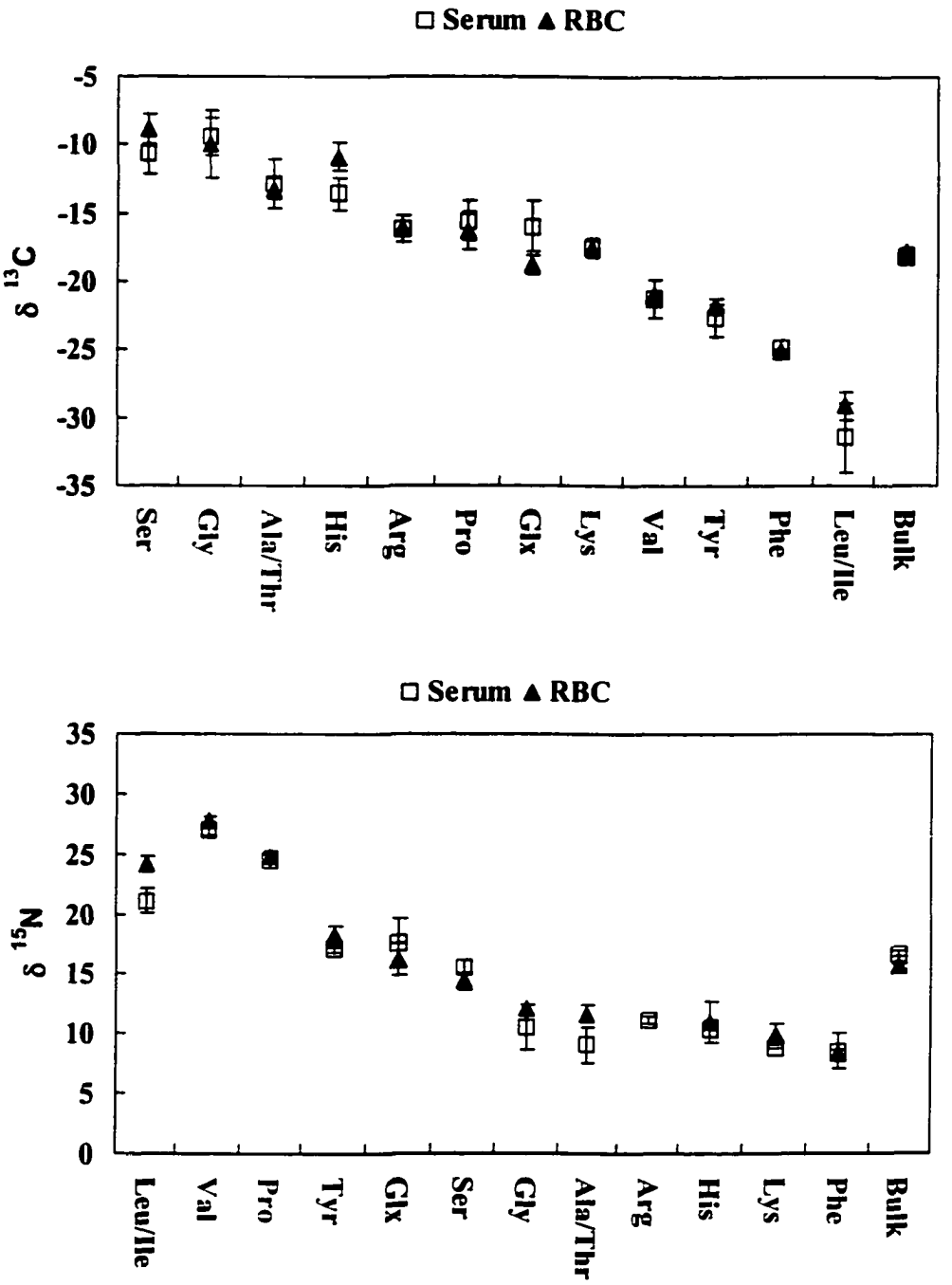


Fig. 4.2 Comparison of the a) carbon and b) nitrogen isotopic compositions of individual amino acids between serum proteins and RBC proteins in captive harbor seals.

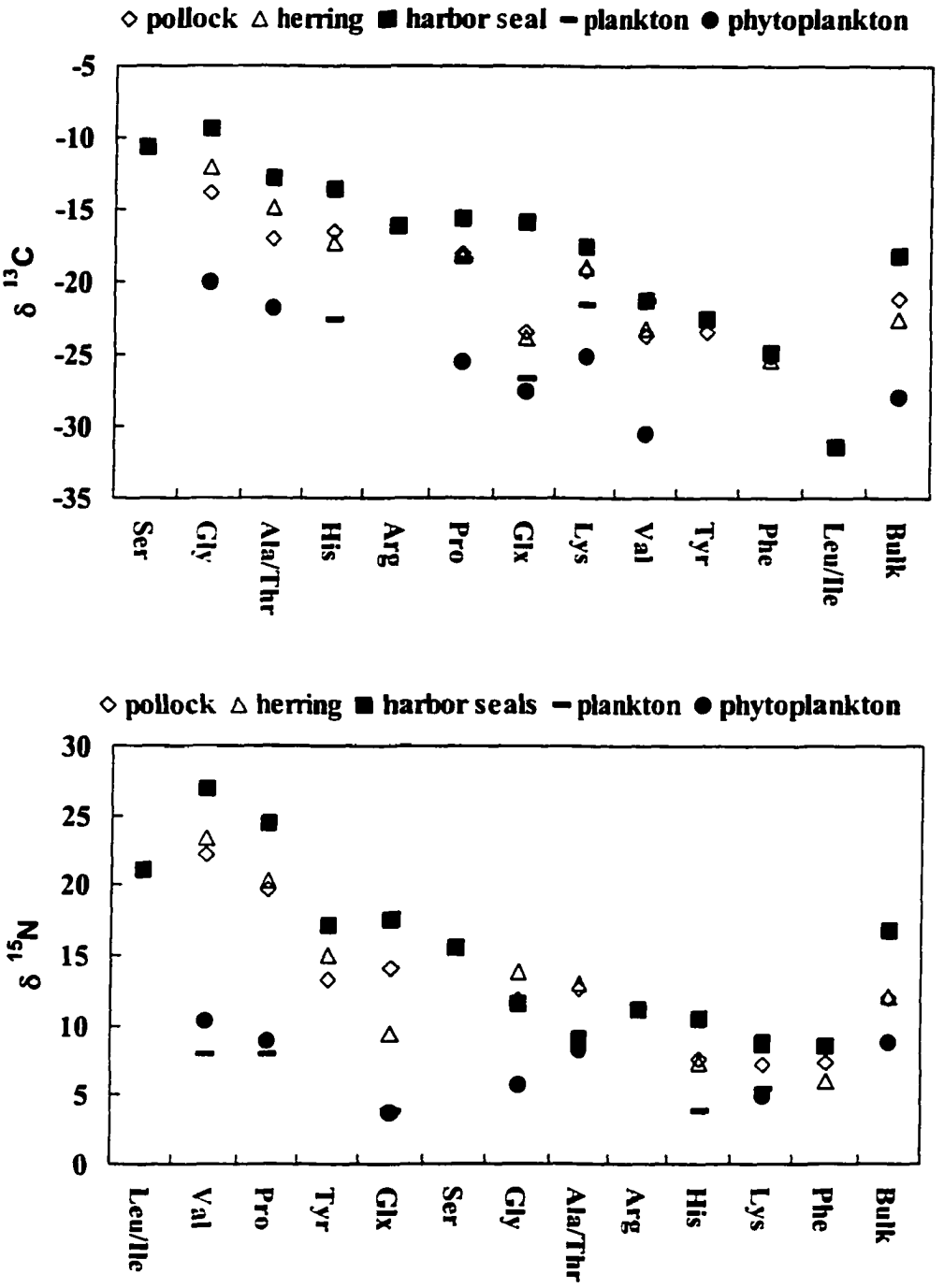


Fig. 4.3 The a) carbon and b) nitrogen isotopic compositions of individual amino acids in several marine organisms.

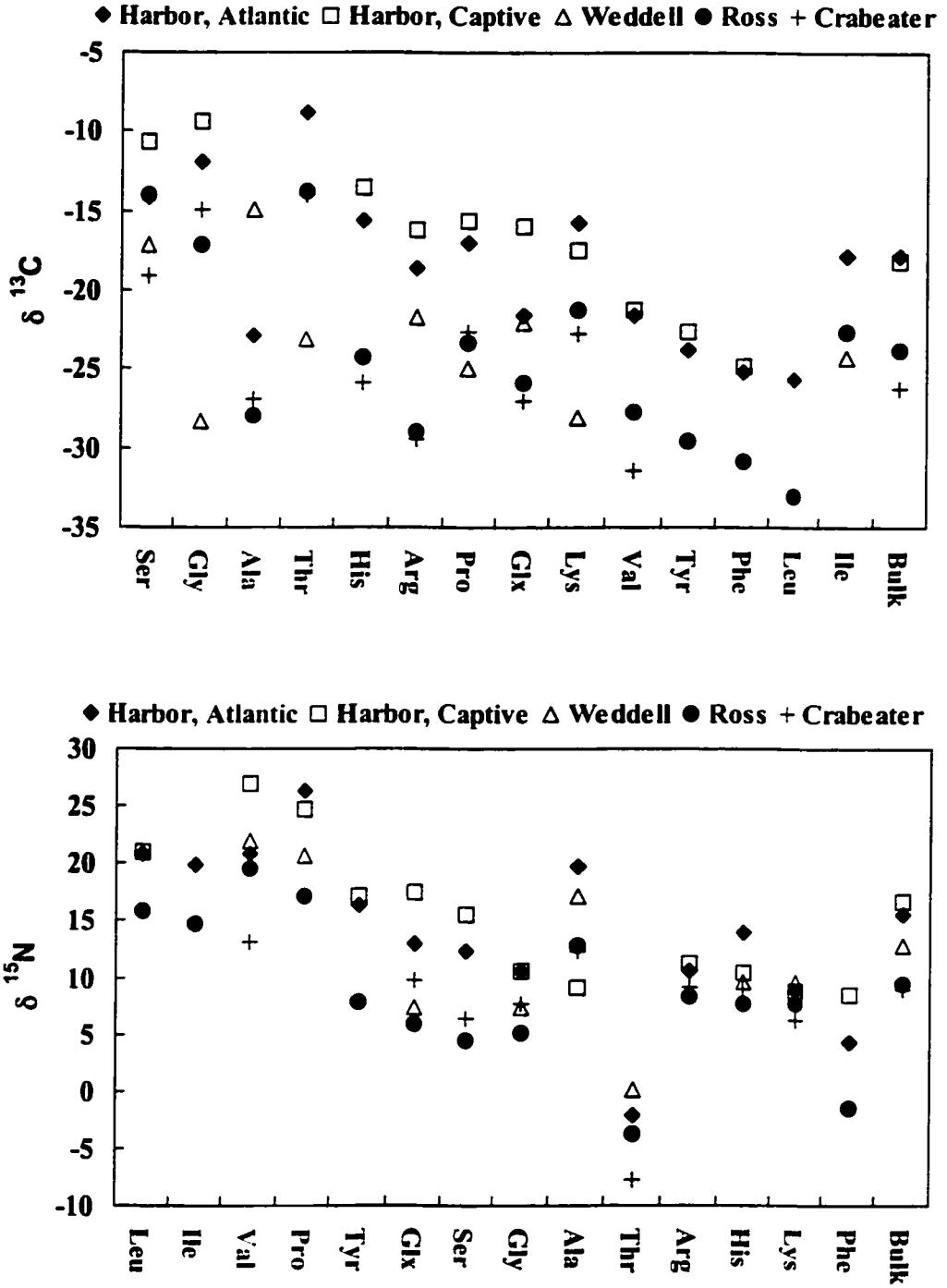


Fig. 4.4 The a) carbon and b) nitrogen isotopic compositions of individual amino acids in serum proteins of wild phocids. Each point is a mean of two measurements.

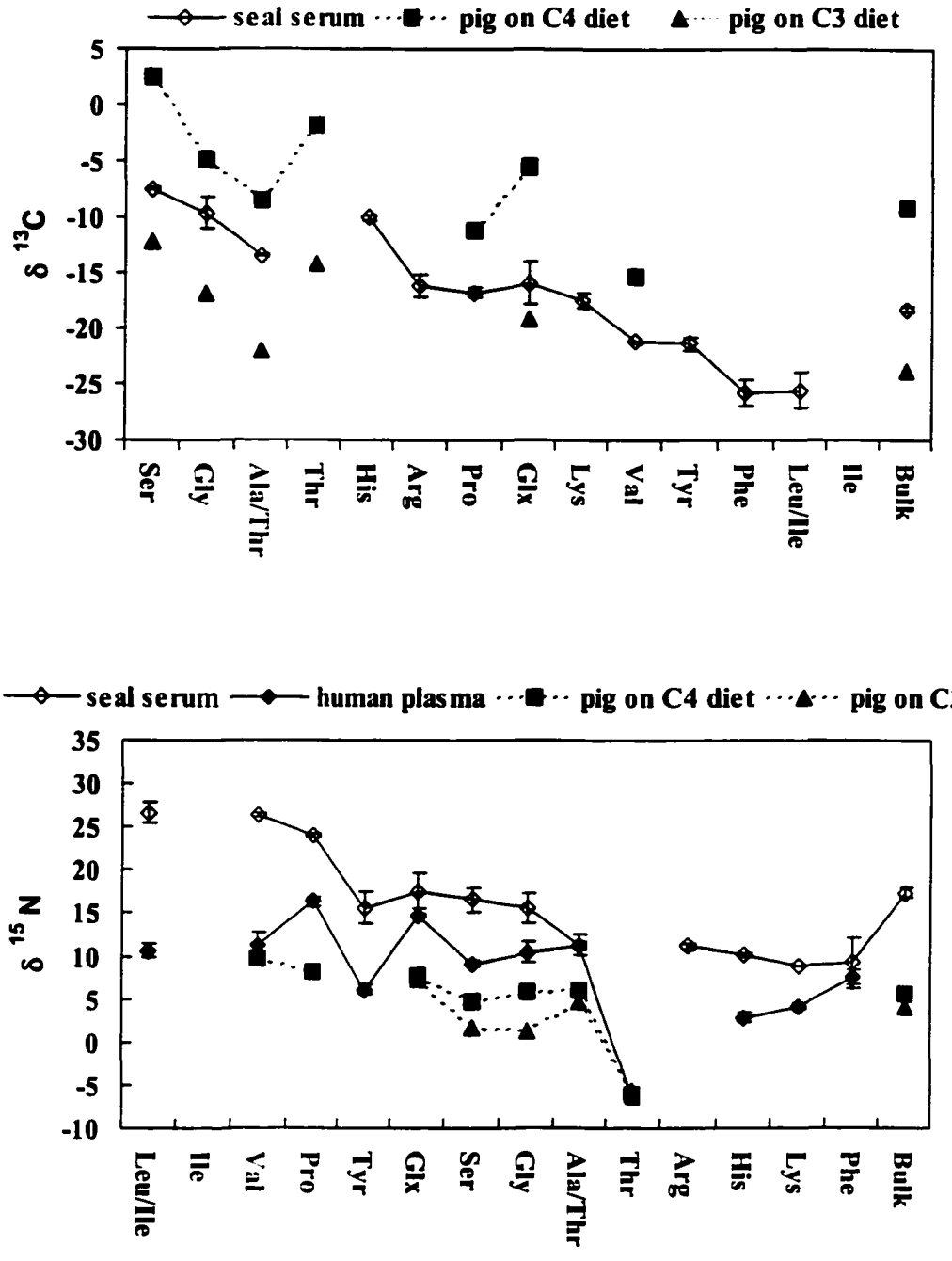


Fig. 4.5 Comparison of the a) carbon and b) nitrogen isotopic compositions of individual amino acids with the literature. Pig data are from Hare et al. 1991, human plasma data are from Petzke et al. 1997.

data are reported for threonine, due to frequent partial co-elution with alanine. Some amino acids, such as valine, tyrosine, leucine and isoleucine, are enriched in ^{15}N but depleted in ^{13}C. In contrast, threonine is extremely depleted in ^{15}N but enriched in ^{13}C. Proline has both high nitrogen and carbon isotope ratios. These distributions of natural abundance amino acid carbon and nitrogen isotope ratios are generally in good agreement with other studies in rat liver proteins (Gaebler et al 1966), bone collagen of pigs (Hare et al. 1991) and human plasma free amino acids (Petzke et al. 1997), as shown in Fig. 4.5.

Trophic enrichments in individual amino acids

Stable isotope techniques have been widely used for tracing the organic matter transfer within food webs for over three decades. This approach is based upon the observation that the carbon and nitrogen isotopic compositions of an organism are similar to or very predictably from those of its food sources (Ostrom and Fry 1993). One of the earliest attempts to investigate trophic enrichments at the molecular level were measurements of the nitrogen isotope ratios in amino acids of the liver protein of the rats raised on different known diets (Geabler et al. 1966). Their data showed that nitrogen trophic enrichments occurred in every rat liver amino acid relative to their dietary proteins (Fig. 4.5). However, few studies of this kind have been done thus far, due to the technical difficulties mentioned earlier.

As shown in Fig. 4.1, most amino acids in serum proteins of captive harbor seals are enriched in both ^{15}N and ^{13}C relative to their fish diet. The enrichments in amino acid ^{15}N are more pronounced than those in amino acid ^{13}C, reflecting the large effects of

transamination and oxidative deamination processes on amino acid nitrogen isotope ratios. These results match the commonly observed differences in trophic enrichments in bulk nitrogen (3 - 4‰) and carbon (0 -1‰) (reviewed by Michener and Schell 1994). However, the magnitude of these offsets in $\delta^{13}C$ and $\delta^{15}N$ values among different amino acids is not uniform, reflecting their different metabolic pathways.

Conservative transfer of AA $\delta^{13}C$ values from the base of food webs

When comparing the amino acid isotopic compositions among species at different trophic levels, as shown in Fig. 4.3, the carbon isotopic ratios are strikingly parallel to each other, with the overall offset consistent with that of their bulk tissues. As shown in Fig. 4.1, the magnitude of the offsets in amino acid $\delta^{15}N$ values along the trophic ladder is generally larger than that in amino acid $\delta^{13}C$, indicating that the metabolic effects on amino acid isotopic fractionation are more pronounced for $\delta^{15}N$ than $\delta^{13}C$. The almost identical patterns in relative carbon isotopic composition of individual amino acids along the trophic ladder indicate the inherently conservative transfer of amino acid $\delta^{13}C$ from primary producers to top predators. Therefore it is not surprising to find similar patterns, but lower absolute values, in amino acid $\delta^{13}C$ in the three Antarctic seal species (Fig. 4.4), indicating that amino acid isotopic compositions, and thus the initial amino acid biosynthetic pathways of phytoplankton, are similar worldwide. The small differences in amino acid carbon isotope ratios among the crabeater seals, Ross seals and Weddell seals probably reflect their presumed dietary differences and trophic status (Laws 1984; M. Castellini, unpublished data). The consistent patterns in relative amino acid carbon

isotopic composition, from herbivores to top carnivores in these marine species from widely separated geographic regimes, imply that the amino acid carbon isotope ratios are set during the initial amino acid biosynthesis by phytoplankton (Hare et al. 1991). These characteristic isotope ratios are transferred to heterotrophic species along the food chains in a predictable manner, but more or less isotopic fractionation may occur due to metabolic modifications. The distinct shifts in absolute amino acid isotope values between the three Antarctic seal species and the harbor seals from the North Pacific and Atlantic are due to geographic differences in stable isotope ratios of primary producers.

Large geographic gradients in isotopic composition exist within marine ecosystems (Saupe et al 1989; Dunton et al. 1989; Schell et al. 1998). For example, phytoplankton in the Antarctic Ocean have particularly low $\delta^{13}C$ (-26.9‰) and $\delta^{15}N$ (0.5‰) values (Wada et al. 1987), due to much slower cell growth rates resulting from light or iron limitation and low water temperature, compared with their counterparts in the high latitude North. Hence, much lower amino acid carbon and nitrogen isotope ratios were found in Weddell, Ross and crabeater seals from the Antarctic Ocean than in harbor seals from the North Pacific and Atlantic Oceans (Fig. 4.4). The characteristic amino acid isotope ratios of phytoplankton determine the isotopic compositions of organisms at higher trophic levels in a specific geographic location because of the transfer of amino acid isotope ratios from phytoplankton to top predators. Studies have shown that carbon isotope ratios of primary producers are shaped by many oceanographic factors. These include light intensity, ambient CO_2 concentrations, phytoplankton growth rates and cell

geometry, and water temperature (Rau et al. 1989; Laws et al. 1995; Rau et al. 1997; Popp et al. 1998; Popp et al. 1999). On the other hand, nutrient availability and nitrogen assimilation pathways result in differing nitrogen isotopic composition of phytoplankton between high latitudes of the Northern Hemisphere and the Antarctic ocean (Altabet and Francois 1994a, b; Montoya 1994).

Metabolic modifications of AA $\delta^{15}N$ values along the trophic ladder

Similar patterns in amino acid nitrogen isotope ratios are also evident along the trophic ladder (Fig. 4.3). However, the magnitude of the offsets is generally more variable for amino acid $\delta^{15}N$ than $\delta^{13}C$, and larger between herbivores (zooplankton) and carnivores (fishes and seals) than within carnivores, especially for some amino acids such as valine, proline and glutamate plus glutamine. These different offsets imply that the metabolic effects on isotopic fractionation are not uniform for each amino acid. Lysine, histidine and phenylalanine usually show lesser variations than non-essential amino acids and branched-chain amino acids, suggesting relatively conservative transfer of nitrogen isotope ratios, with lesser metabolic modification by transamination or deamination.

As discussed earlier, the characteristic isotope ratios in primary producers initialize the amino acid carbon and nitrogen isotope values of a given ecosystem. When these basal isotope values are transferred to foraging species at higher trophic levels, metabolic processes in consumers modify these baseline ratios to different degrees, dependent upon metabolic pathways followed by a given amino acid. Seasonal

physiological changes and changes in dietary protein sources or intake may also affect isotopic fractionation for a given amino acid (Hare et al. 1991).

Several researchers have hypothesized that the pathways of amino acid catabolism play a key role in the magnitude of isotopic fractionation (Sick et al. 1997). Large fractionations occur if the amino acid is metabolized via transamination or oxidative deamination (Gaebler et al. 1966; Macko et al. 1987). Essential amino acids cannot be biosynthesized by the organisms themselves, but must be supplied from dietary proteins. If the intake of these essential amino acids is not much greater than requirements for growth and maintenance, a higher portion of dietary essential amino acids will be used for protein synthesis, instead of participating in transamination or oxidative deamination. Hence, it is reasonable to expect that isotopic signatures of essential amino acids will be conserved to a greater degree, as observed in our study. Stable isotope signatures of essential amino acids may act as conservative natural tracers in food web studies.

CONCLUSION

The following conclusions can be reached based on this study. a) Amino acids vary more in their carbon and nitrogen isotopic composition within an organism than across trophic levels. The differences in isotopic composition among amino acids reflect their distinct biosynthetic pathways. b) The magnitude of $\delta^{15}N$ trophic enrichments is not uniform for different amino acids, indicating the varying metabolic effects on a given amino acid. c) The magnitude of nitrogen isotopic fractionation in lysine, histidine and

phenylalanine is usually much smaller than that in branched-chain and most non-essential amino acids, reflecting more conservative transfer of the above essential amino acids along the food chains. Some essential amino acids can thus be used as natural biomarkers to provide more detailed information on the food sources or habitat usage in studies of foraging ecology. d) The strikingly similar patterns in relative amino acid carbon isotopic composition of organisms at different trophic levels indicate conservative transfer of amino acid carbon from primary producers to top predators. e) Differences in amino acid $\delta^{13}C$ values between phocids from the North Pacific or Atlantic and their counterparts from the Antarctic reflect geographic variations of isotope ratios in phytoplankton.

Chapter 5

Metabolic Effects on Amino Acid Isotope Ratios in Captive Harbor Seals (*Phoca vitulina*) Revealed by Infusion of ^{15}N-labeled Amino Acid Tracers

ABSTRACT

The metabolic effects on isotopic composition of individual amino acids were investigated in captive harbor seals (*Phoca vitulina*) by infusion of ^{15}N-labeled amino acid tracers. Each ^{15}N-labeled amino acid tracer (glycine, phenylalanine, leucine and valine) was infused intravenously as a bolus dose into a given captive harbor seal, and the transfer of ^{15}N label from the amino acid tracer to other amino acids was monitored in serum and red blood cells (RBCs) over time. The differing effects of transamination or oxidative deamination reactions on individual amino acids was estimated by measuring amino acid ^{15}N enrichments using reversed-phase HPLC separation and subsequent IRMS measurement of carbon and nitrogen isotope ratios in individual, underivatized amino acids. Results showed that amino acid metabolic pathways governed the patterns of amino acid ^{15}N enrichments following different tracer infusions. It is evident from all four tracer infusions that some essential amino acids, including phenylalanine, threonine, lysine and histidine, were little affected by transamination or deamination processes. These essential amino acids therefore might be useful as natural conservative "biomarkers" to track specific food sources or habitat usage, if the isotopic compositions of the food sources were distinctly different. The variation of ^{15}N enrichments over time differed considerably between serum and RBC proteins following the tracer infusions,

with the serum proteins rapidly acquiring the label and the RBC proteins showing a much slower incorporation, consistent with their differing turnover rates. The turnover curves for serum fitted a two-pool model for every infused amino acid tracer.

Key words: ^{15}N-labeled amino acid tracers; harbor seals; *Phoca vitulina*; transamination; deamination; metabolic isotopic effects.

INTRODUCTION

The complex interactions among biochemical, physiological and ecological processes make it difficult to interpret stable isotopic patterns in consumers based solely on bulk stable isotope ratios in whole animal tissues (Gannes et al. 1997). Stable isotopic analysis of individual amino acids may give a more precise view of feeding ecology, by providing insight into metabolic isotopic effects occurring during food assimilation and metabolism. The goal of this study is to identify if there are any essential amino acids whose isotope ratios are not modified by metabolic processes, and to assess quantitatively the effects of transamination or oxidative deamination reactions on amino acid isotopic compositions. The extent to which amino acids are isotopically conservative was investigated by the infusion of ^{15}N-labeled amino acid tracers into captive harbor seals and subsequent measurements of ^{15}N enrichments in individual amino acids. Since essential amino acids come exclusively from dietary proteins for some organisms, and isotopic fractionation mainly occurs during transamination or oxidative deamination processes (Gaebler et al. 1966; Macko et al. 1987), if there is little or no excess intake,

little isotopic fractionation will occur due to metabolic modifications. Isotope ratios in these amino acids then can be used as conservative "biomarkers" to track specific prey species or habitat usage.

The infusion of amino acid tracers enriched with stable isotopes represents a powerful tool in protein metabolic studies in humans (Wolfe 1992; Meier-Augenstein 1999; El-Khoury 1999). However, the common analytical techniques in such studies involve derivatization of the amino acids and subsequent gas chromatography–combustion-isotope ratio mass spectrometry (GC-C-IRMS) (Metges and Petzke 1999; Meier-Augenstein 1999). These techniques are complex and require the correction of results for the added moiety used to make the amino acids volatile. This study employed a technique using reversed-phase HPLC separation of underivatized amino acids on a semi-preparative column. The subsequent IRMS measurement of underivatized amino acids makes it practical to obtain both carbon and nitrogen isotope ratios in a single run, at either natural abundance or enrichment levels. By monitoring amino acid ^{15}N enrichments in serum and RBC protein hydrolyzates of captive harbor seals following each tracer infusion, transamination or deamination effects on different amino acids can be measured and conservative amino acids identified.

MATERIALS AND METHODS

One of four ^{15}N-labeled amino acid tracers (glycine, phenylalanine, leucine or valine) was infused into one of the three captive harbor seals. Amino acid tracer

experiments provide two data sets: time course of ^{15}N enrichments in individual amino acids and transfer of ^{15}N label from tracer amino acids to other serum or RBC protein amino acids. For instance, variations of glycine ^{15}N enrichments in serum and RBC proteins over time represent a) a rapid ^{15}N label loss diluted by whole body glycine tissue pools and b) a relatively rapid incorporation of ^{15}N label into serum proteins, but much slower incorporation of ^{15}N label into RBC proteins, from the liver ^{15}N labeled pool, following the ^{15}N-labeled glycine infusion. Measurement of ^{15}N enrichments in other serum and RBC protein amino acids, such as serine, alanine and glutamate plus glutamine, following ^{15}N-labeled glycine infusion, may provide insight into differential partitioning in transamination or deamination reactions of these amino acids. Refer to ^{15}N-labeled Amino Acid Tracer Experiments, Harbor Seal Blood Sampling and Stable Isotope Analysis and Stable Isotope Analysis of Individual Amino Acids in Chapter 2 for the detailed methods.

RESULTS

Nitrogen turnover in serum and RBC proteins

Nitrogen isotope ratios or ^{15}N enrichments were monitored in bulk serum proteins for 282 days following the ^{15}N-labeled glycine infusions into the three captive seals (Fig. 5.1a). The highest serum $\delta^{15}N$ values were observed in all three seals sampled at 4 hrs following the infusions. The serum $\delta^{15}N$ values subsequently decreased exponentially, and returned to a level that was indistinguishable from the pre-injection values at approximately 120 days, as shown in Fig. 5.1a. Data analysis showed that ^{15}N

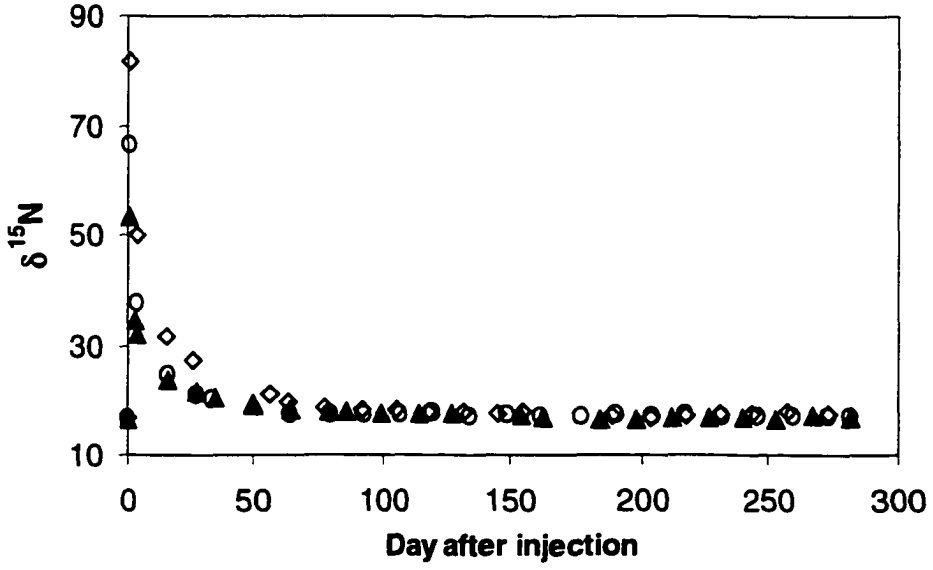


Fig. 5.1a The $\delta^{15}N$ variations in serum proteins of the three captive harbor seals following the ^{15}N-labeled glycine infusions. Initial serum samples were taken approximately 4 hours following injection. ◇Pender; ▲Snapper and ○ Poco.

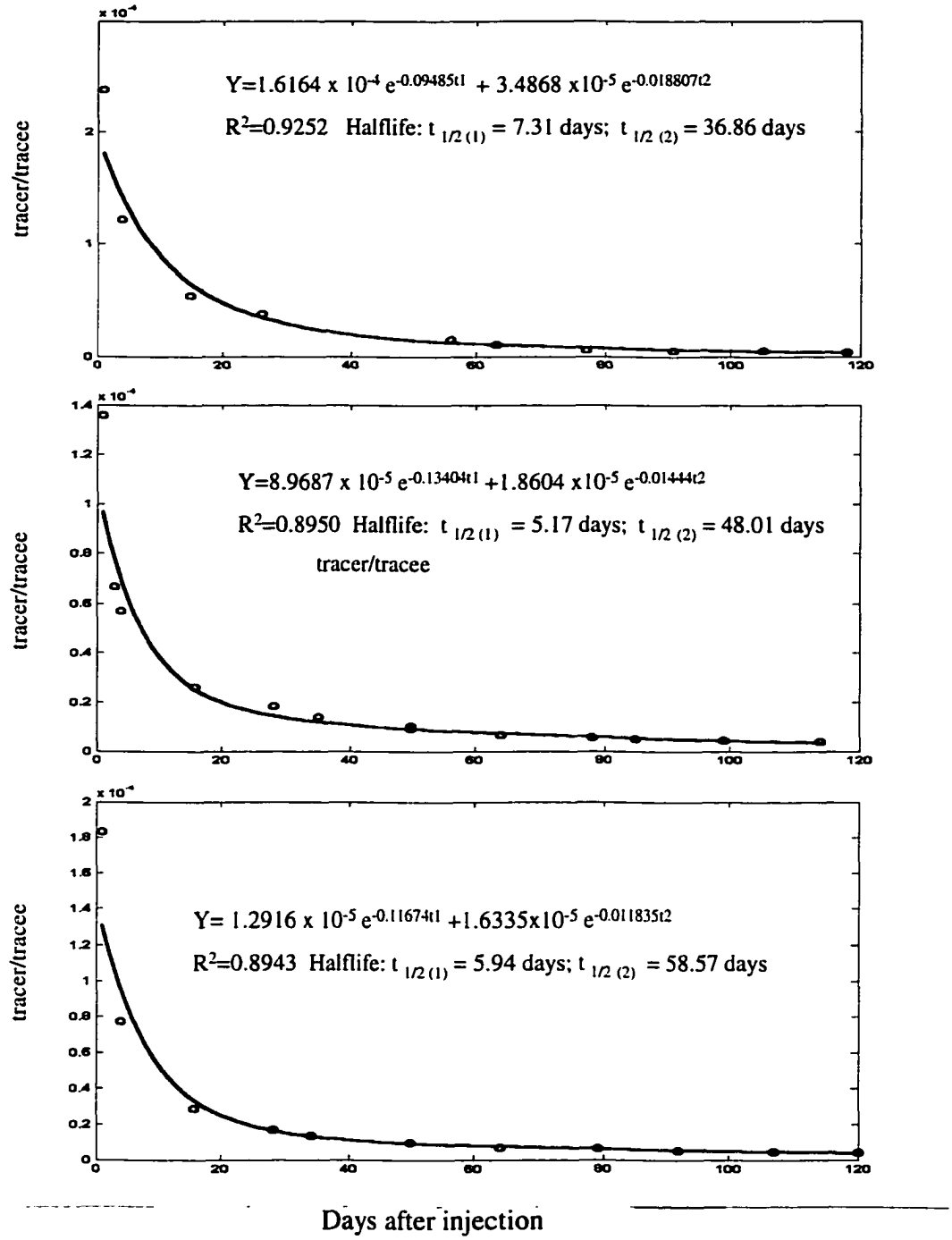


Fig. 5.1b. Curves fitted using a two-pool model. Data used here are the first 120 days of Fig.5.1a. The $\delta^{15}N$ values of Y-axis are converted to the ratios of tracer/tracee. Top: Pender; middle: Snapper; bottom:Poco.

enrichments in bulk serum proteins of all three seals conformed to a two-pool model, as shown in Fig. 5.2 and expressed by the equation: $y = C_A e^{-\alpha t1} + C_B e^{-\beta t2}$. In the equation y = ^{15}N enrichment at the tracer/tracee ratio, which is analogous to the specific activity used with a radioactive tracer, and is the most appropriate expression of stable isotope enrichment data when performing kinetics calculations with a tracer; t1, t2 = time elapsed after tracer infusion (day); and C_A, C_B, α, β are constants (Goodman and Noble 1968; Wolfe 1992). Curve fitting showed that approximately a 120-day monitoring period after the tracer infusion may provide the best results (Fig.5.1b), comparable to turnover studies of human plasma cholesterol (Goodman and Noble 1968). Table 5.1a, b summarizes the kinetic parameters calculated using the two-pool model, including the half-lives of pool A ($t_{½(1)}$) and pool B ($t_{1/2(2)}$); the size of pool A (M_A); the rate constant for removal of total enriched label from pool A (K_{AA}) and pool B (K_{BB}); and the production rate in pool A (PR_A).

Nitrogen isotope ratios or ^{15}N enrichments were also monitored in bulk RBC proteins for 282 days following the ^{15}N-labeled glycine infusions into the three captive seals. As shown in Fig. 5.3, changes of $\delta^{15}N$ values in RBC proteins over time differed considerably from those in serum proteins. The ^{15}N label appeared in RBCs at a much lower level and slower rate. Unlike serum proteins whose $\delta^{15}N$ values dropped to 30%-40% of their maximum within the first 30 days following the tracer infusions (Fig. 5.1a), $\delta^{15}N$ values in RBCs on day 4 had reached only half of the maximum ^{15}N label found on day 16. The high $\delta^{15}N$ values in RBCs (up to 25.8‰ in Poco) sampled at 4 hrs after the

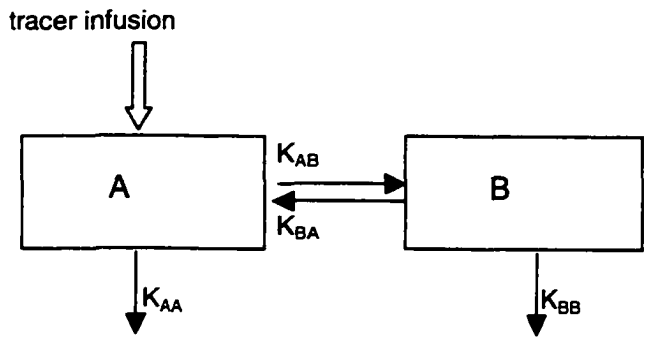


Fig.5.2 Schematic representation of a simple two-pool model with tracer injection and irreversible loss occurring from pool B. Rate constants are denoted by the K values. Refer to Goodman and Noble 1968.

Table 5.1a Information on the Dec. 1998 tracer experiments and the kinetic parameters calculated using a two-pool model

	Pender	Snapper	Poco
1st tracer experiments			
Age/Sex	2.5/Male	15/Male	23/Female
Body weight (kg) [a]	45	86	60.5
Dosage	Glycine	glycine	glycine
Dose injection amount (mmol)	6.581	6.581	6.581
AA percentage in serum [b]	5.80 ± 0.17	5.80 ± 0.17	5.80 ± 0.17
AA percentage in RBCs [b]	7.93 ± 0.25	7.93 ± 0.25	7.93 ± 0.25
Maximum label in serum (‰)[c]	81.67	53.41	66.60
Maximum label in RBCs (‰)[c]	20.02	19.74	20.12
C_A	1.6164×10^{-4}	8.9687×10^{-5}	1.2916×10^{-4}
C_B	3.4868×10^{-5}	1.8604×10^{-5}	1.6335×10^{-5}
α (day^{-1})	-0.09485	-0.1340	-0.1167
β (day^{-1})	-0.018807	-0.014444	-0.01184
$t_{1/2\ (1)}$ first exponential (days)	7.31	5.17	5.94
$t_{1/2\ (2)}$ second exponential (days)	36.86	48.01	58.57
K_{AA}	-0.0814	-0.1135	-0.1049
K_{BB}	-0.0323	-0.0350	-0.0236
M_A (g)	492.28	893.30	664.88
PR_A (g/day)	27.19	49.42	38.91

a. Body weights were measured right before the tracer infusion.
b. Mean ± SD of mole percent of the injected amino acid tracer in serum or RBC proteins.
c. Measured $\delta^{15}N$ values in serum or RBC proteins.

Table 5.1b Information on the June 2000 tracer experiments and the kinetic parameters calculated using a two-pool model

	Pender	Snapper [d]	Poco
2nd tracer experiments			
Age/Sex	4/Male	16/Male	24/Female
Body weight (kg) [a]	43.0	84.0	56.5
Dosage	phenylalanine	leucine	valine
Dose injection amount (mmol)	6.497	7.998	8.922
AA percentage in serum [b]	5.35 ± 0.17	11.76 ± 0.15	9.32 ± 0.19
AA percentage in RBCs [b]	6.72 ± 0.23	13.65 ± 0.31	11.44 ± 0.39
Maximum label in serum (‰)[c]	48.14	36.86	61.96
Maximum label in RBCs (‰)[c]	21.67	22.66	21.40
C_A	8.1966×10^{-5}		1.4923×10^{-4}
C_B	4.5168×10^{-5}		3.353×10^{-5}
α (day^{-1})	-0.10376		-0.078569
β (day^{-1})	-0.028331		-0.021915
$t_{1/2\,(1)}$ first exponential (days)	6.68		8.82
$t_{1/2\,(2)}$ second exponential (days)	24.47		31.63
K_{AA} (day^{-1})	-0.0770		-0.0682
K_{BB} (day^{-1})	-0.0551		-0.0323
M_A (g)	747.69		690.49
PR_A (g/day)	39.87		36.80

a. Body weights were measured right before the tracer infusion.
b. Mean ± SD of mole percent of the injected amino acid tracer in serum or RBC proteins.
c. Measured $\delta^{15}N$ values in serum or RBC proteins.
d. Insufficient data points for curve fitting.

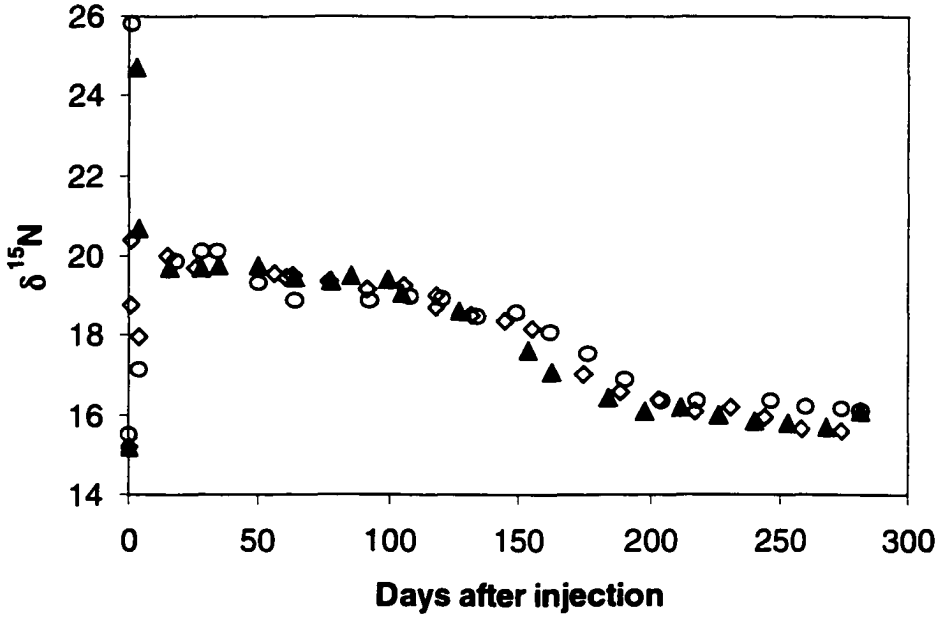


Fig. 5.3 The $\delta^{15}N$ variations in RBC proteins of the three captive harbor seals following the ^{15}N-labeled glycine infusions. ◇Pender; ▲Snapper and ○ Poco.

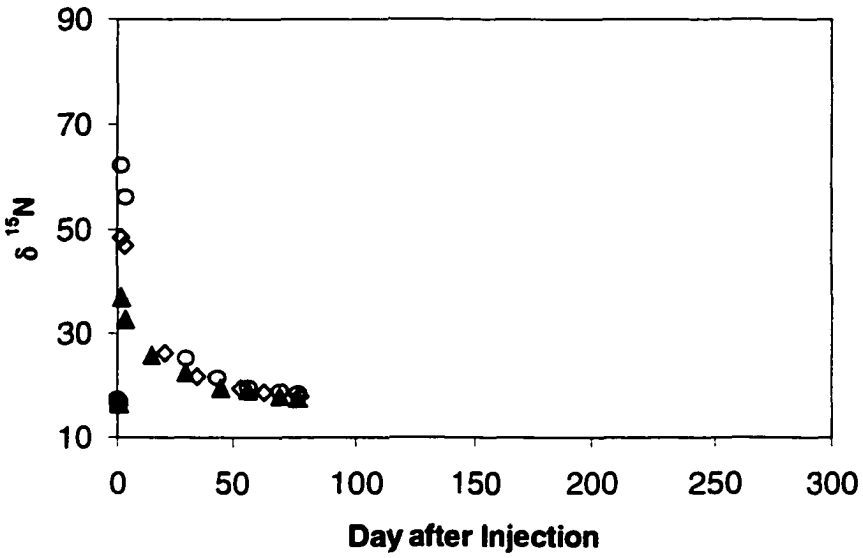
tracer infusions were probably due to contamination by serum left during blood separation, since RBCs were not washed. The $\delta^{15}N$ values in RBCs declined only slightly from 16 to 120 days, then decreased almost linearly over the next 80-100 days to a level that was just slightly higher than that of pre-injection values.

During the second essential amino acid (leucine, valine and phenylalanine) tracer experiment, which lasted for 77 days, similar patterns in serum and RBCs but different levels of ^{15}N label were observed (Fig. 5.4a,b). As shown in Table 5.1b, the levels of ^{15}N label in serum were affected by the dosage injected and the total blood volume of the seals. As expected, lower $\delta^{15}N$ values were observed in the second tracer experiment, because of the lower molar dosages of the larger molecular weight essential amino acids compared with glycine.

Metabolic effects on AA ^{15}N enrichments in serum and RBCs

The appearance of ^{15}N label in amino acids other than the infused tracer amino acid provided insight into the amino acid metabolic pathways or, specifically, transamination or deamination reactions. Different patterns in ^{15}N enrichments of amino acids were observed with the four amino acid tracers. Fig. 5.5 - 5.7 show the variations of amino acid ^{15}N enrichments over time in serum and RBC proteins of harbor seal Pender following the glycine infusion and the phenylalanine infusion, one and half years after the glycine injection. Fig. 5.8 and Fig.5.9 show the amino acid ^{15}N enrichments in serum and RBC proteins of harbor seals Poco and Snapper after injection of the branched-chain

a)



b)

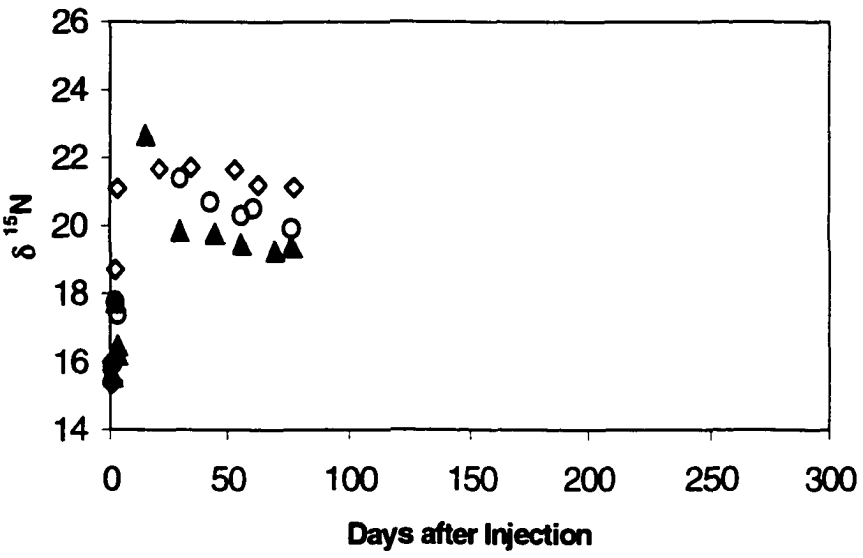


Fig. 5.4 The $\delta^{15}N$ variations in a) serum and b) RBC proteins of the three captive harbor seals during the second amino acid tracer experiment. ◇ Pender, phenylalanine injected; ▲ Snapper, leucine injected and ○ Poco, valine injected.

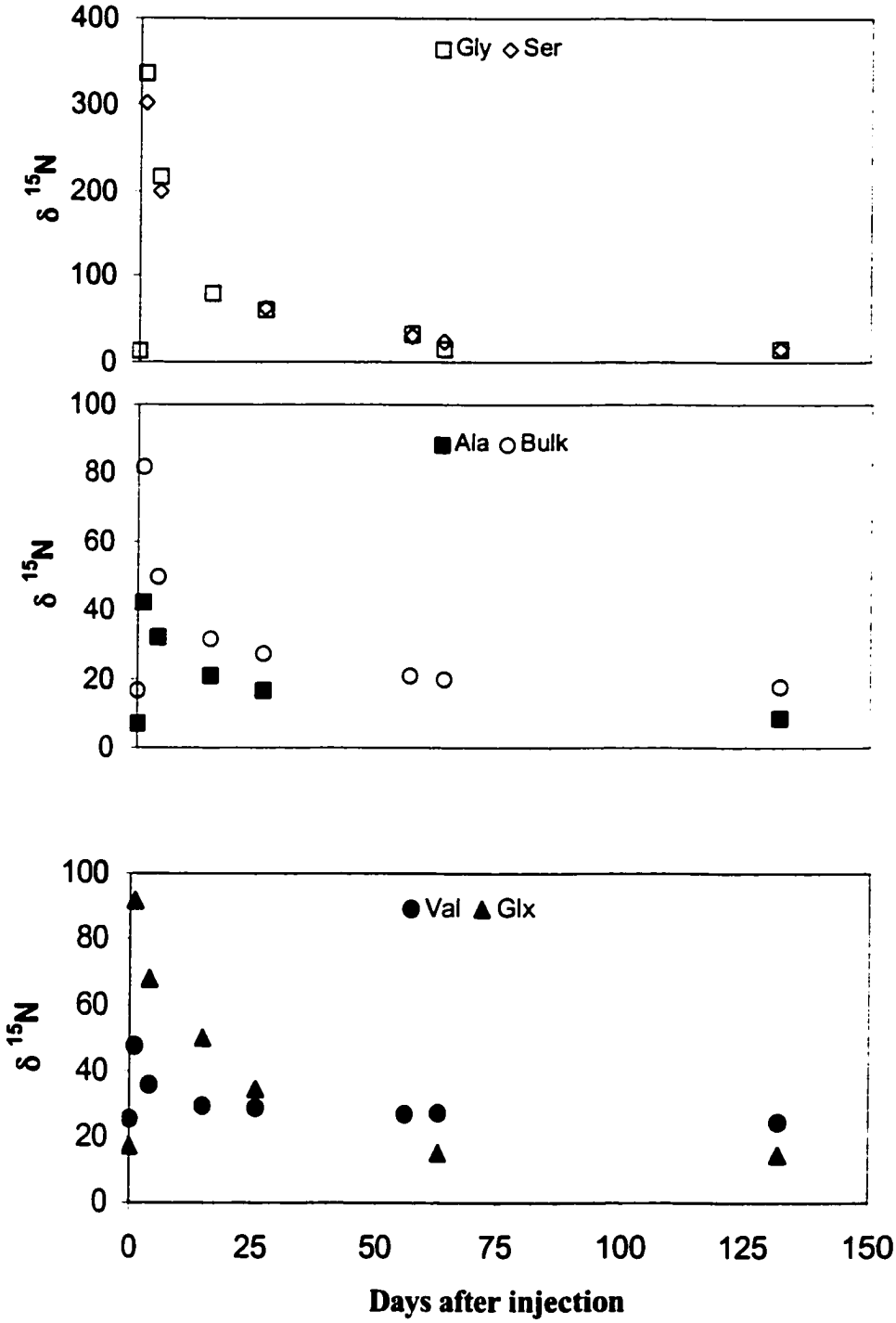


Fig. 5.5a The variations of amino acid ^{15}N enrichments in serum protein hydrolyzates over time following the ^{15}N-labeled glycine infusion into Pender.

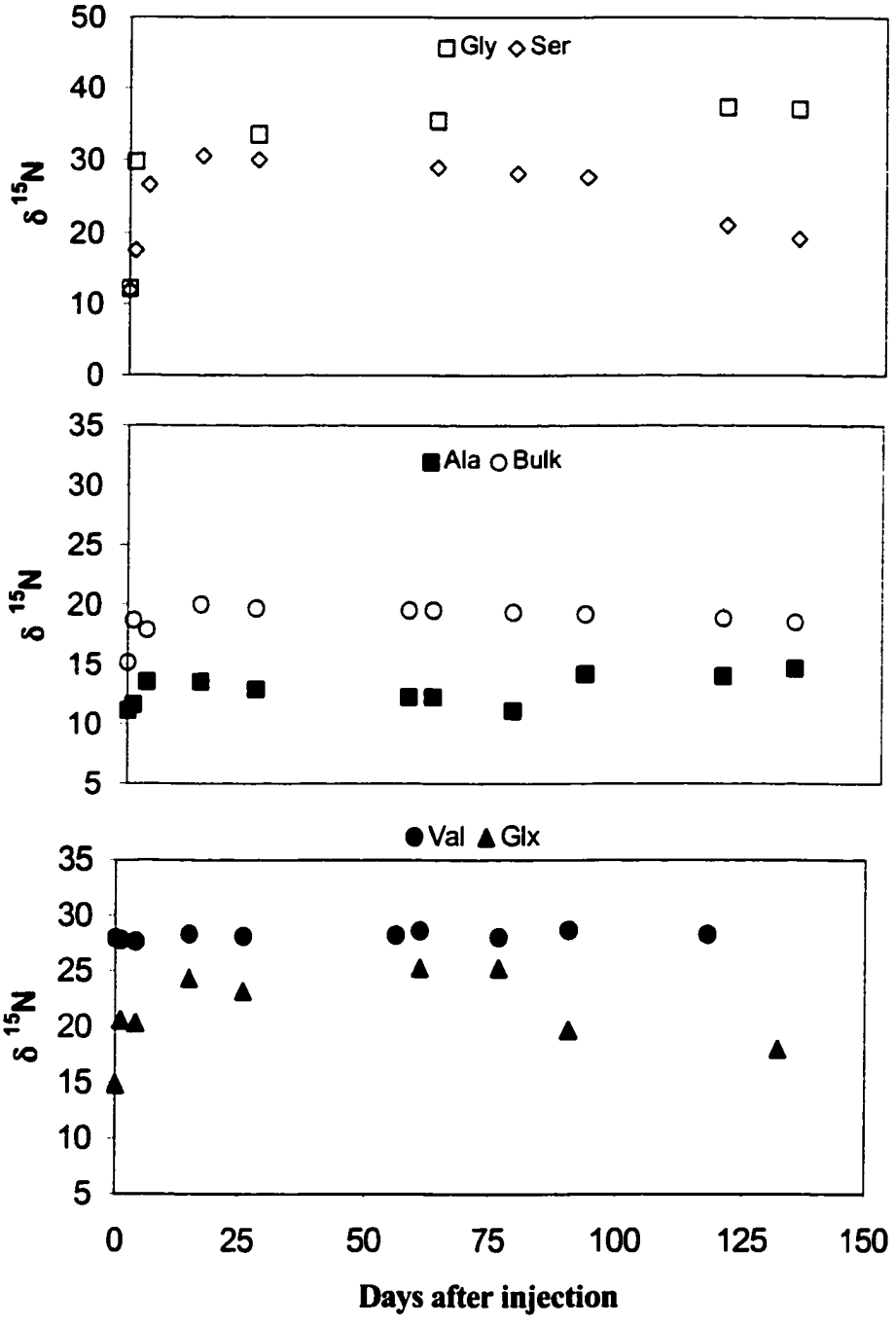


Fig. 5.5b The variations of amino acid ^{15}N enrichments in RBC protein hydrolyzates over time following the ^{15}N-labeled glycine infusion into Pender.

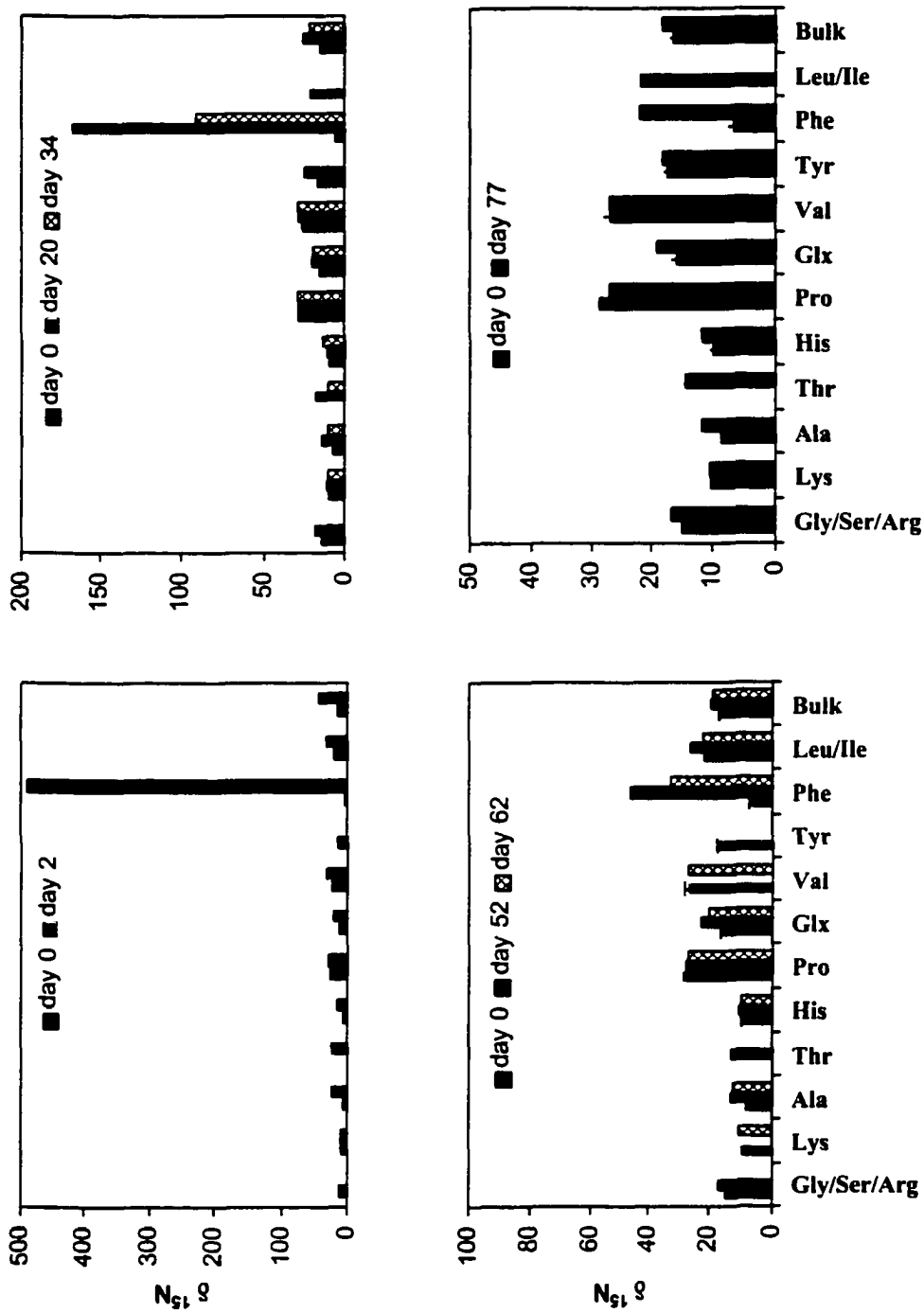


Fig. 5.6a Amino acid ^{15}N enrichments in serum protein hydrolyzates following the ^{15}N-labeled phenylalanine infusion into Pender

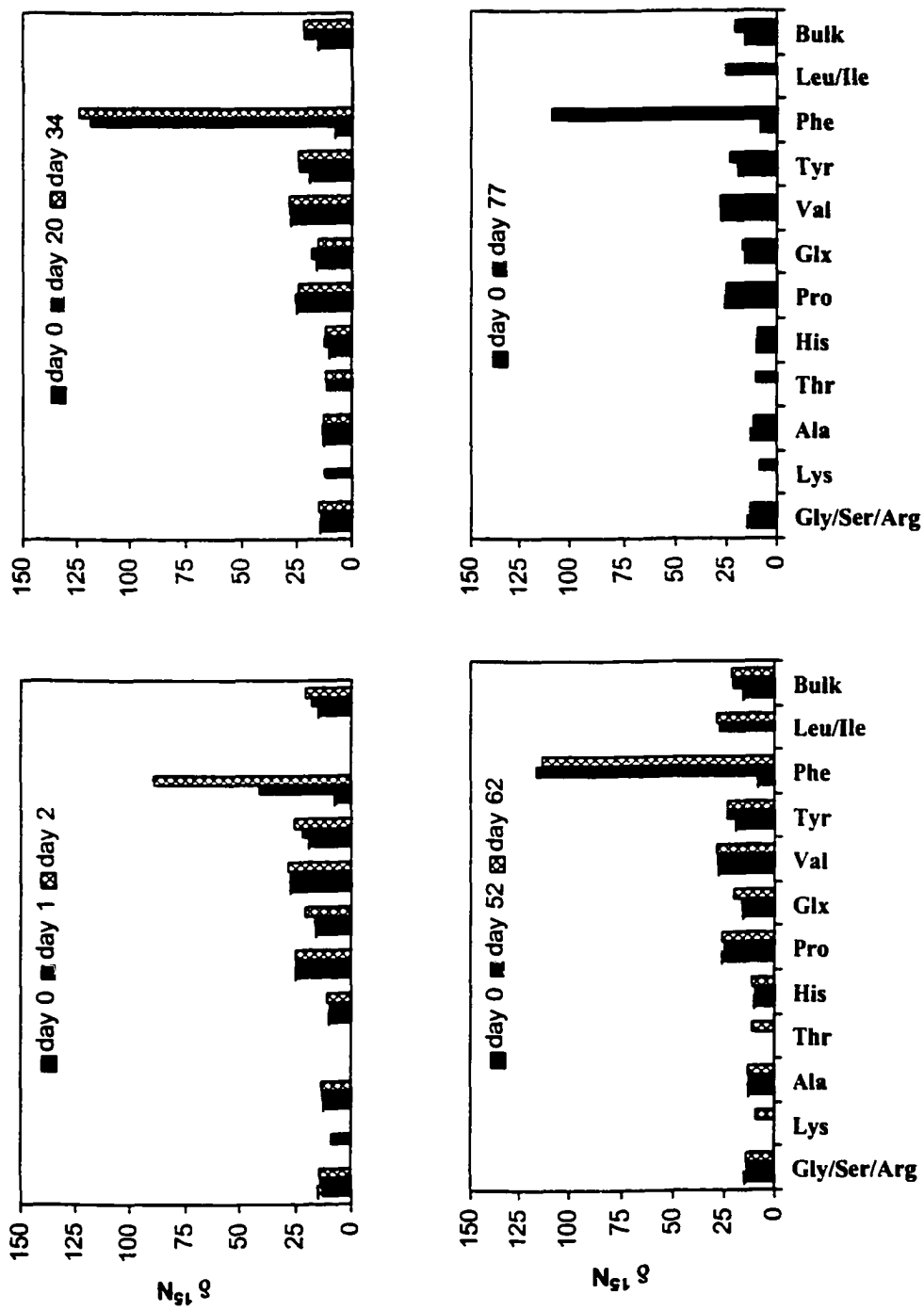
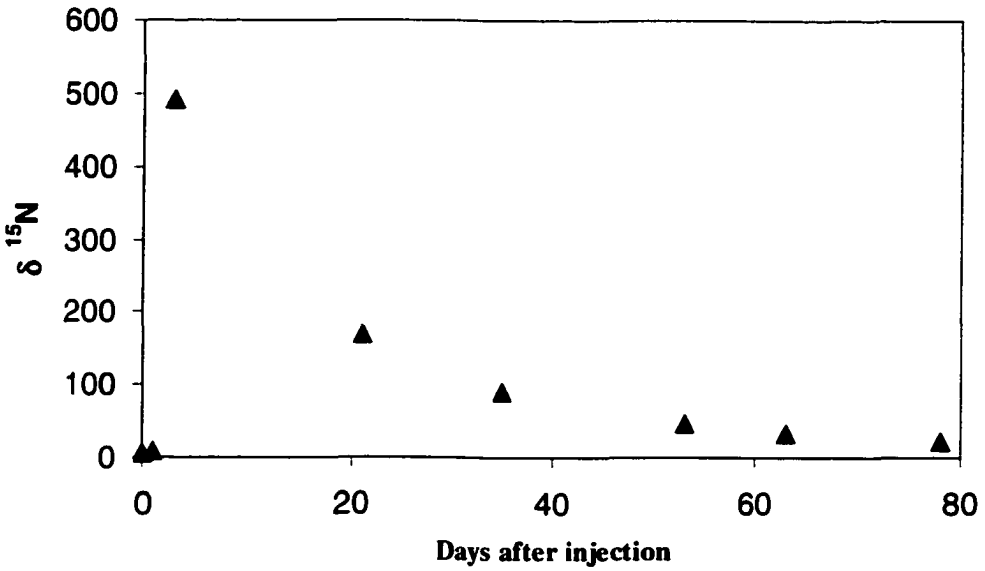


Fig. 5.6b Amino acid ^{15}N enrichments in RBC protein hydrolyzates following the ^{15}N-labeled phenylalanine infusion into Pender

a)



b)

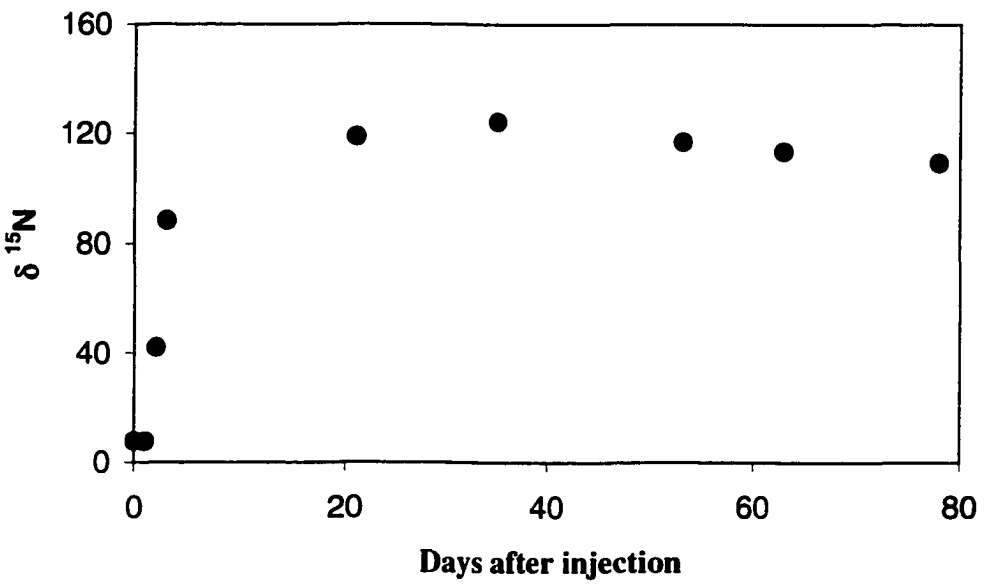


Fig. 5.7 The variations of phenylalanine ^{15}N enrichment in a) serum and b) RBC protein hydrolyzates over time following the ^{15}N-labeled phenylalanine infusion into Pender.

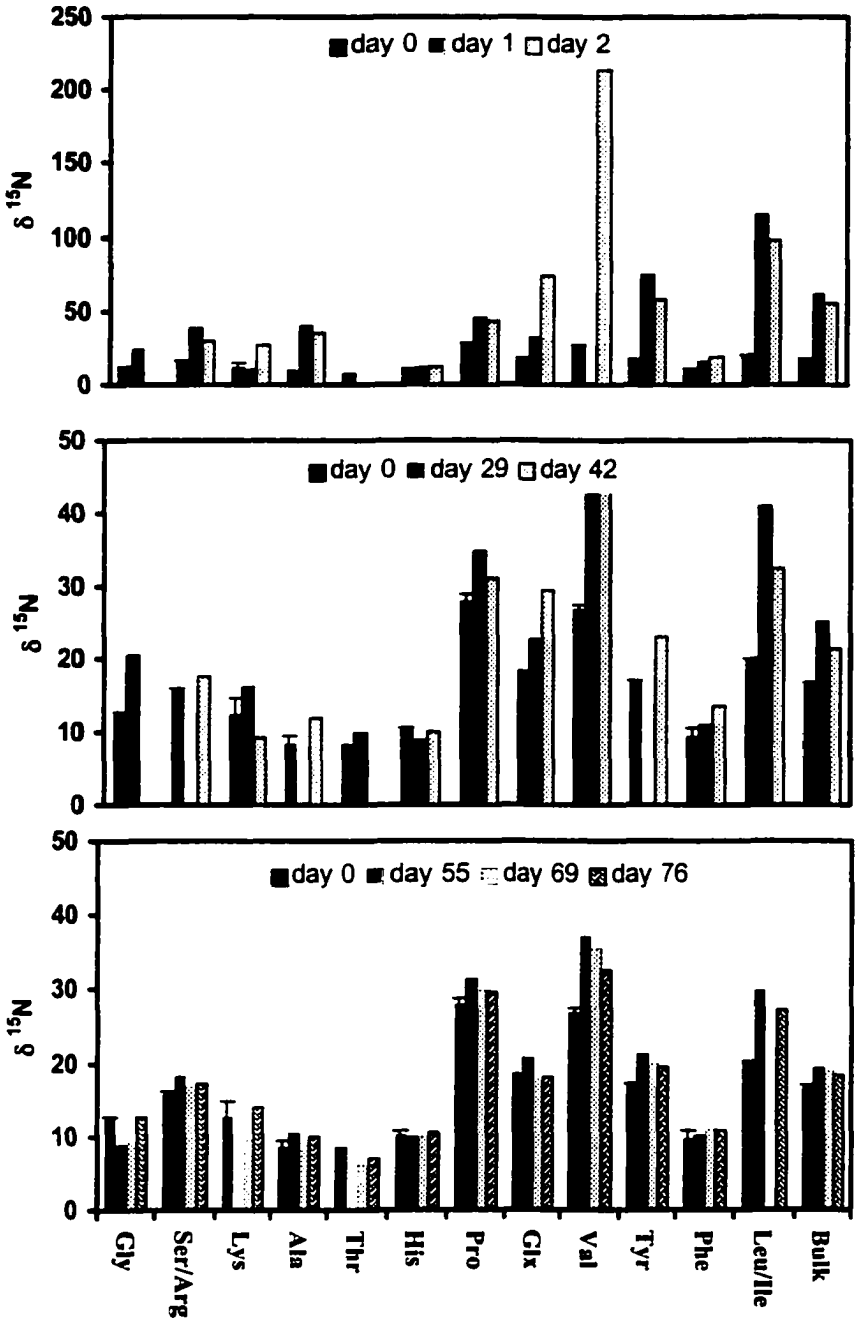


Fig. 5.8a ^{15}N enrichments in amino acids of serum protein hydrolyzates following the ^{15}N-labeled valine infusion into Poco.

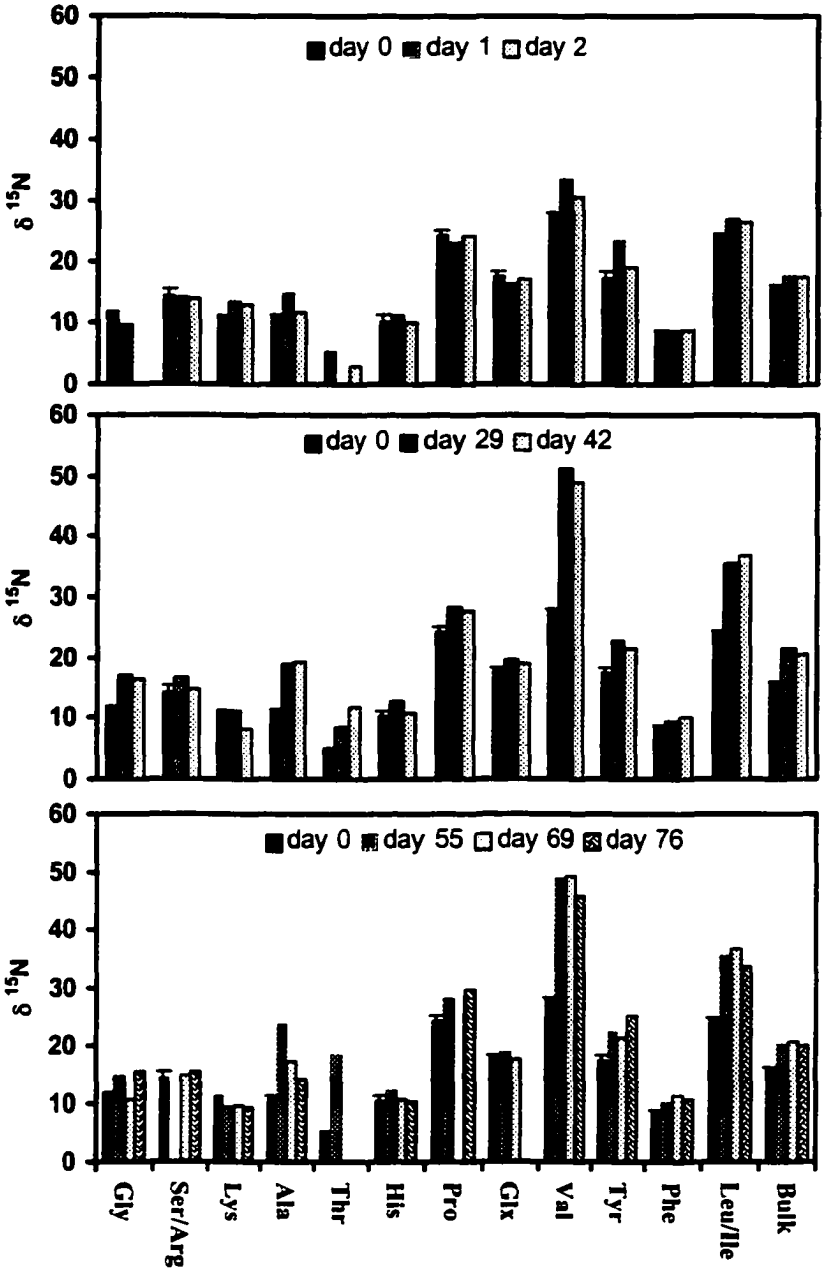


Fig. 5.8b ^{15}N enrichments in amino acids of RBC protein hydrolyzates following the ^{15}N-labeled valine infusion into Poco.

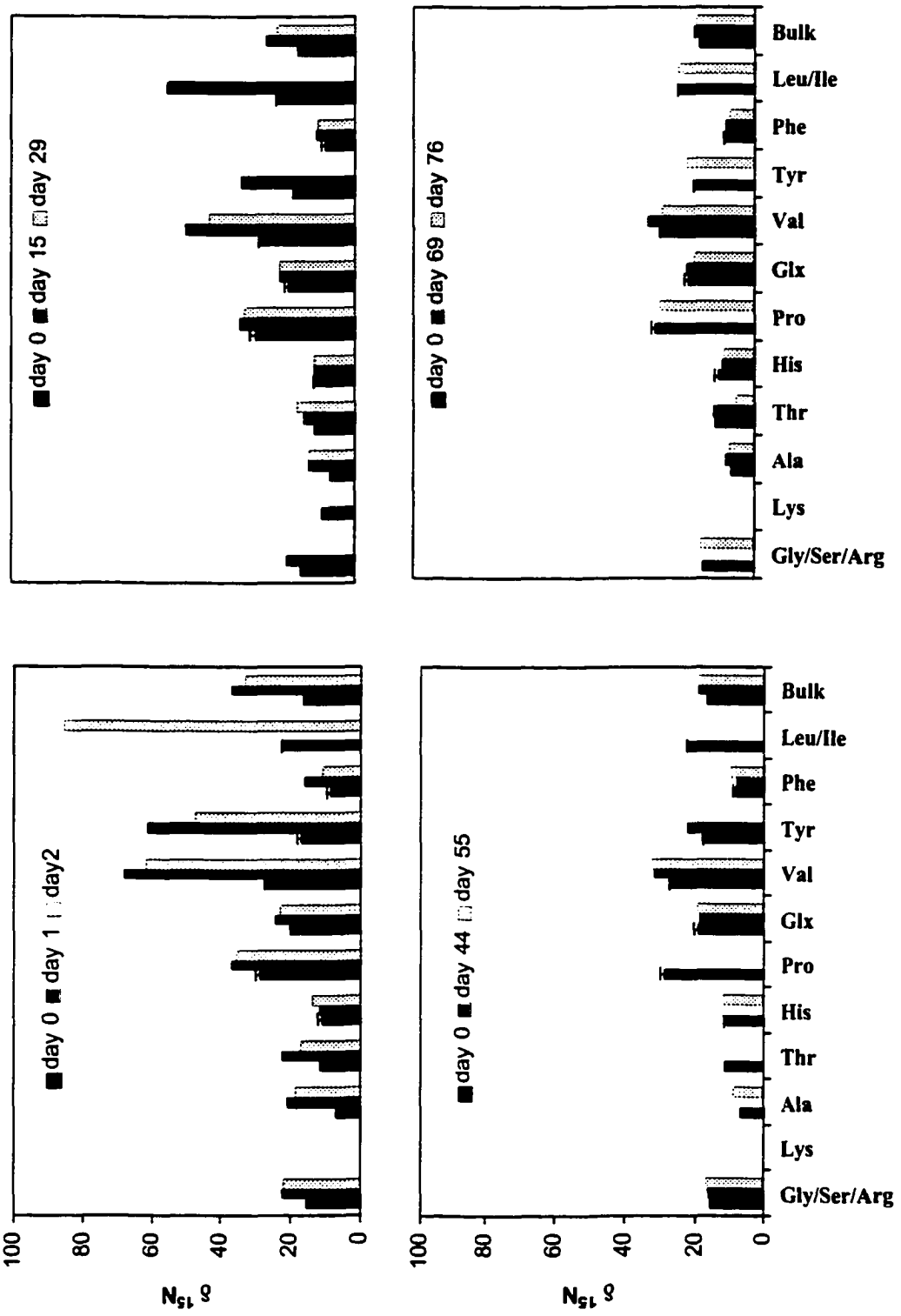


Fig. 5.9a Amino acid ^{15}N enrichments in serum protein hydrolyzates following the ^{15}N-labeled leucine infusion into Snapper.

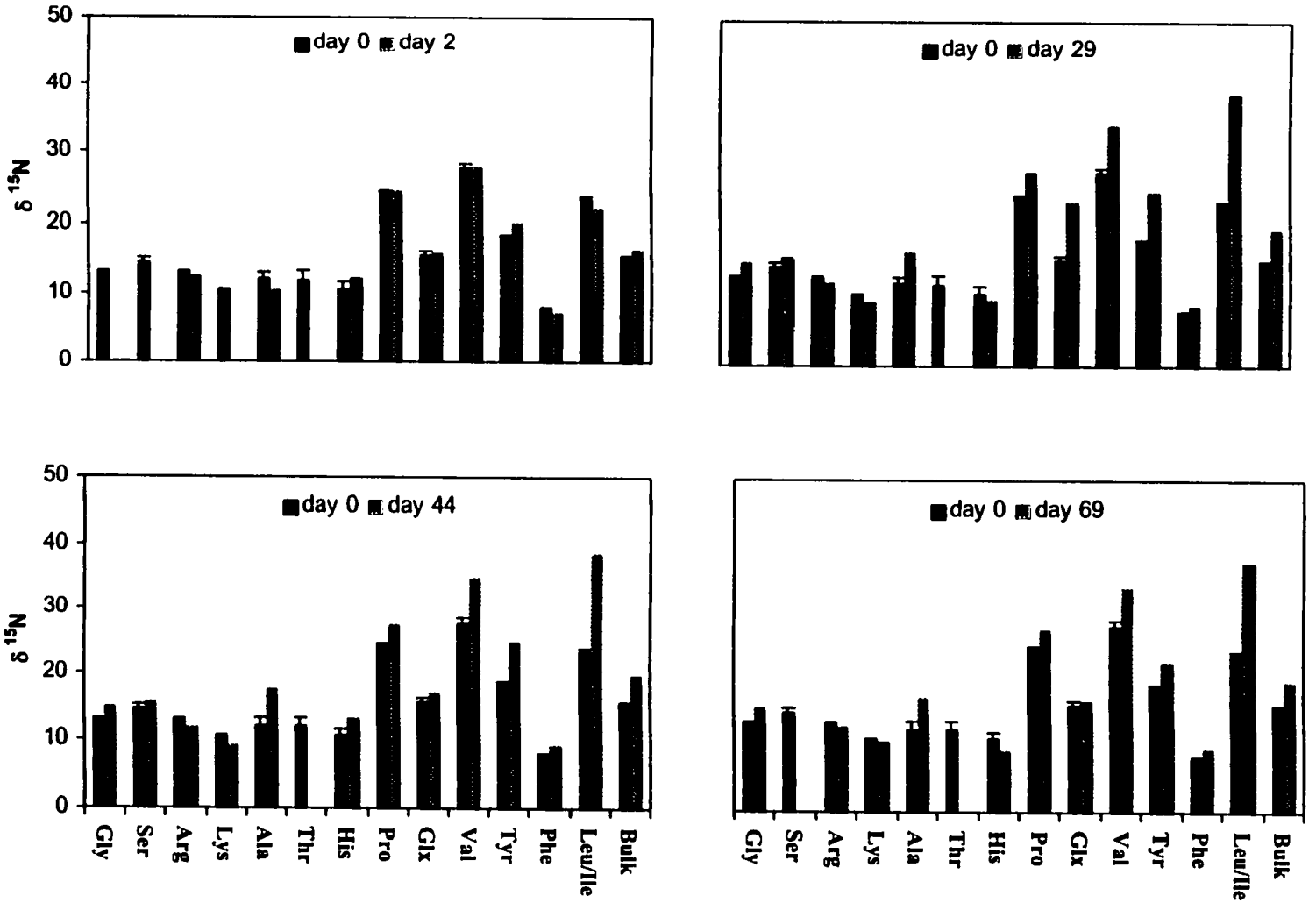


Fig. 5.9b Amino acid ^{15}N enrichments in RBC protein hydrolyzates following the ^{15}N-labeled leucine infusion into Snapper.

amino acids valine and leucine, respectively. Results are not available for all measurable amino acids due to insufficient sample size.

^{15}N enrichments of glycine, serine, alanine, valine, proline, histidine and glutamate plus glutamine in serum and RBC proteins were measured following ^{15}N-labeled glycine infusion. As shown in Fig. 5.5, particularly high ^{15}N enrichments were found in serine (approximately 2000% increase on day 2 compared with the baseline value before injection) and the transaminating amino acids glutamate plus glutamine (500% increase on day 2) and alanine. Significant ^{15}N enrichment was also evident in valine (80% increase on day 2), but little enrichment was found in histidine or proline. Similar patterns of ^{15}N variations over time were observed in serum and RBCs for all ^{15}N enriched amino acids, but as was the case for bulk serum and RBCs, the $\delta^{15}N$ of specific amino acids in RBCs was smaller than that in serum, and the $\delta^{15}N$ of RBC protein amino acids decreased more slowly than that of serum.

Unlike the glycine tracer, only a modest amount of ^{15}N label from ^{15}N-labeled phenylalanine infusion was observed in tyrosine, alanine and glutamate plus glutamine, and no significant ^{15}N enrichments were found in other amino acids (Fig.5.6). As shown in Fig. 5.7, the phenylalanine $\delta^{15}N$ in serum reached 491.7‰ at 28 hrs, which was more than 60 times higher than the phenylalanine $\delta^{15}N$ baseline value before injection, and 10 times higher than that in bulk serum sampled at the same time (48.1‰). Phenylalanine

$\delta^{15}N$ values in RBCs also rose from a baseline value of 7.8‰ to 42.2 ‰ at 28 hrs, and then increased gradually to a maximum value of 124.1‰ on day 35.

Similar patterns in ^{15}N transfers were observed in serum and RBCs of Poco and Snapper, with either ^{15}N-labeled valine or leucine infusion, consistent with the similarity in the metabolic pathways of these two branched-chain amino acids. As shown in Fig. 5.8 and Fig. 5.9, the ^{15}N label from leucine or valine was transferred to many other amino acids by means of transamination or oxidative deamination reactions via the common intermediates. The highest ^{15}N enrichments were found in other branched-chain amino acids due to their interchangeable metabolic pathways. Medium ^{15}N enrichments were observed in alanine, proline and glutamine plus glutamate, and the lowest enrichments in histidine, threonine, lysine and phenylalanine.

DISCUSSION

Turnover curves of serum and RBC proteins

Amino acid metabolism as measured by the infusions of ^{15}N-labeled amino acid tracers is a complex process. Several processes may contribute to the temporal trends of ^{15}N enrichments in serum and RBC proteins. The ^{15}N label from amino acid tracers can be rapidly diluted by a) equilibrium with body free amino acid pools; b) exchange between various body protein pools and c) loss, irreversibly, via catabolic end products such as urea and ammonia during protein breakdown (Matthews et al. 1981). However, the temporal changes in ^{15}N enrichment in serum proteins can be described by a two-pool

model, although the second amino acid tracer experiments were probably too short to derive accurate parameters.

Based on the two-pool model, two kinetically distinguishable pools or compartments are involved in the turnover of serum protein ^{15}N. The first exponential decay represents a rapid exchange between free amino acids, body proteins and catabolic end products following tracer infusions, suggesting a relatively slower incorporation of ^{15}N label into serum albumin. Our data showed that more than 90% ^{15}N label was no longer in the bloodstream when the first blood sample was taken at 4 hrs following the infusions, based upon the estimation of "expected" initial ^{15}N enrichments from the dose and harbor seal serum pool size, if it was assumed that ^{15}N-labeled free amino acid tracers incorporated exclusively into serum proteins. Loss of label from serum could occur as a consequence of a rapid exchange of the blood free amino acid ^{15}N label with many other body protein pools, especially with muscle, the largest body protein pool, or an irreversible loss of blood free amino acid ^{15}N label as urea via catabolic pathways. The second exponential decrease may reflect the turnover of the total body exchangeable serum protein pool (Wolfe 1992). However, one should realize that the pool in question does not have a precise physical meaning, but is actually composed of a number of metabolically heterogeneous pools in various tissues (Goodman and Noble 1968).

RBCs have different turnover curves than serum, with a much lower ^{15}N label, but retaining the label for almost 100 days before it noticeably decreases. Since bones are

continually producing new blood cells to replenish the body supply, the initial increase in $\delta^{15}N$ of RBCs reflects the partial replacement of new red blood cells synthesized from the ^{15}N-labeled bone marrow nitrogen pool. The appearance rate and the level of ^{15}N label in RBCs depend on the extent to which the bone marrow nitrogen pool acquires ^{15}N label during the exchange between various body protein pools following the tracer infusion, and how fast newly synthesized RBCs incorporate the ^{15}N label into the RBC protein pool. The later linear decline, beginning about 120 days following tracer infusion, is due to the fact that RBCs formed near the time of tracer infusion started to die and new RBCs synthesized from an unlabeled ^{15}N pool gradually replaced the old RBCs. This time period reflects the approximate life cycle of the RBC protein in harbor seals, which is consistent with the average life 120 day span of a red blood cell in humans. In other words, approximately 200 days are probably required for ^{15}N label of red blood cell proteins to completely turn over. This is in good agreement with some previous results in birds that RBC isotopic composition represents diet integrated over several months (Hobson and Clark 1992a).

Metabolic effects on amino acid ^{15}N enrichments in serum and RBCs

The two hypothetical extremes following ^{15}N-label amino acid tracer infusions are: no ^{15}N enrichments in any amino acids other than the tracer itself, or ^{15}N is incorporated rapidly and equally into all amino acids. However, in most experiments, partial incorporation of ^{15}N label is observed (Matthews et al. 1981). The varying patterns of amino acid ^{15}N enrichments following different amino acid tracer infusions reflect the

metabolic pathways of different amino acids. For the glycine tracer infusion, the extremely high degree of ^{15}N enrichment in serine compared with other amino acids is due to the rapid interconversion of glycine and serine during their metabolism (Matthews et al. 1981). Significant ^{15}N enrichment in glutamate results from the incorporation of ^{15}N ammonia into glutamate via α-ketoglutarate, with the ^{15}N ammonia coming from ^{15}N enriched glycine or serine breakdown. Alanine shows almost the same ^{15}N enrichment as glutamate, because both glutamate and alanine serve as carriers of amino groups between peripheral tissues and the liver. Transamination reactions via pyruvate and α-ketoglutarate can transfer amino groups from glutamate to alanine (Petzke et al. 1997). Since glycine is not primarily metabolized by transamination, only minor ^{15}N enrichments in most other amino acids are expected (Matthews et al. 1981). Following ^{15}N-labeled valine or leucine infusion, the highest ^{15}N enrichment is immediately observed in other branched-chain amino acids due to their interchangeable metabolic pathways. ^{15}N label from these branched-chain amino acids can incorporate into glutamate by transamination via the same intermediates. Although proline does not participate in transamination reactions, it can be synthesized directly by ring closure of glutamate (Lehninger et al. 1993). Therefore ^{15}N enrichment in proline should be similar to that of glutamate. The generally low enrichments in lysine and threonine are because both amino acids follow irreversible pathways of oxidation, without transamination, in mammalian tissues (Metges et al. 1996). Likewise, phenylalanine does not participate into transamination and is metabolized by conversion to tyrosine (Matthews et al. 1981), consistent with the extremely high ^{15}N enrichment found in phenylalanine, compared

with similar dosages of valine and leucine tracers. This result indicates that phenylalanine may be used as a relatively conservative tracer, without much transamination occurring during its metabolism.

Essential amino acids as conservative "biomarkers"

By infusions of ^{15}N-labeled amino acid tracers in our experiments and subsequent measurements of ^{15}N enrichments in the tracer amino acid and other individual amino acids in serum and RBC proteins, the metabolic pathways of both tracer amino acids and most other protein amino acids can be elucidated. By measuring ^{15}N enrichment in the tracer amino acids, glycine, phenylalanine, valine and leucine, following tracer infusion, the hypothesis that the isotopic composition of these tracer amino acids is conserved during incorporation into serum and RBC proteins was tested. By measuring ^{15}N enrichment in other protein amino acids following tracer infusion, the isotopic effects of transamination or oxidative deamination on other protein amino acids were assessed. By reviewing amino acid ^{15}N enrichment data obtained from four tracer infusions, it is evident that essential amino acids, phenylalanine, threonine, lysine and histidine, are generally involved in only minor transamination and their isotopic compositions are more conservative than those of other amino acids, including non-essential and branched-chain amino acids. These essential amino acids may thus act as conservative "biomarkers", although they can also be modified by oxidative breakdown if there is an excess intake from diet. Therefore, only if an amino acid comes exclusively from diet and nearly all of

the intake is used for protein synthesis, will its isotopic signature be conserved in the tissue proteins of the consumer (Sick et al. 1997).

CONCLUSION

The metabolic pathways control changes in the isotopic composition of a given amino acid following tracer infusions. The extent to which the given amino acid is involved in transamination or deamination reactions can be examined by measuring ^{15}N enrichments in tracer amino acid and other protein amino acids using reversed-phase HPLC separation and subsequent IRMS measurement of carbon and nitrogen isotope ratios in individual, underivatized amino acids. This technique is especially useful in studies of protein metabolism using stable isotope tracers. The preliminary results of tracer experiments and the kinetic inferences using a two-pool model provide a foundation for future study of harbor seal protein metabolism, which has not been explored thus far. The modest involvement in transamination of some essential amino acids, elucidated by this study, may make them useful in studies of foraging ecology. Natural abundance isotopic compositions in these relatively, isotopically conservative essential amino acids may provide more specific indicators of prey consumption or feeding locations.

Chapter 6

Amino Acid Composition in Phocids and the Variations of Serum Free Amino Acids in Captive Harbor Seals in Response to Dietary Protein Intake

ABSTRACT

Serum free amino acids (FAA) and protein hydrolyzable amino acids (HAA) in serum and red blood cells (RBCs) of captive harbor seals (*Phoca vitulina*) were monitored during the course of a two-year controlled feeding trial. Elevated serum free glutamine and alanine levels were observed in captive harbor seals when the diet was switched from Pacific herring (*Clupea pallasi*) to walleye pollock (*Theragra chalcogramma*). In contrast, the levels of serum free branched-chain amino acids, valine, leucine and isoleucine, and several other essential amino acids, lysine, arginine and threonine, decreased in response to the diet switching. HAA in serum and RBC proteins had almost constant composition, reflecting the consistent composition of their constituent proteins, independent of dietary protein source or intake. Comparisons of HAA profiles in fish tissue protein hydrolyzates between herring and pollock revealed that their mole percent compositions were similar, although total HAA concentrations (THAA, μmol/g dry weight) in pollock were on average 1.5 times higher than those in herring. FAA and HAA profiles were compared among four seal species: captive harbor seals in the controlled feeding trial, wild harbor seals from Prince William Sound and southeast Alaska, wild harbor seals from the North Atlantic, and Weddell seals (*Lepthonychotes weddellii*), Ross seals (*Omatophoca rossii*) and crabeater seals (*Lobodon*

carcinophagus) from the Antarctic. In some seal species, notable differences in serum FAA composition were observed, presumably linked to the diversity of their diets. However, HAA profiles in serum proteins were found to be indistinguishable among these phocid species.

Keywords: amino acid concentration; dietary protein intake; phocids; protein metabolism

INTRODUCTION

Harbor seals (*Phoca vitulina*) are small pinnipeds that are widely distributed throughout the coastal regions of the Northern Hemisphere. The populations of harbor seals in Alaska have declined significantly over the past two decades, particularly in Prince William Sound (Pitcher 1990; Frost et al. 1994) and the western Gulf of Alaska (Small and DeMaster 1995). The population decline is apparently continuing even today, and may also be occurring in the eastern Bering Sea, although the populations in southeast Alaska remain stable (Small 1996). One hypothesis is that a change in harbor seal diet occurred during the late 1970's in response to a climate shift that co-occurred with a change in forage fish species (Niebauer and Hollowed 1993). Before ~1978, Pacific herring were more abundant, but later, walleye pollock predominated (Anderson and Piatt 1999). As top predators, marine mammals play important roles in the top-down control of community structures in marine ecosystems. The decreasing carrying capacity of the Gulf of Alaska and Bering Sea ecosystems, thought to be caused by the climate-

driven regime shift and its bottom-up effects, may be contributing to concurrent population fluctuations among marine mammals, birds and forage fishes in western Alaska (Niebauer and Hollowed 1993; Beamish 1993; Schell et al.1998).

Although protein metabolism and dietary requirements for essential amino acids in humans and many terrestrial mammals have been actively investigated for many years, there are few previous studies on marine mammals (Metges et al. 1999). Due to the concern that some fish diets may cause nutritional stress in harbor seals, serum free amino acid composition (FAA) of captive harbor seals was monitored as an important indicator of nutrition and health status during a controlled feeding trial. Effects of the amino acid composition of dietary protein and harbor seal daily body protein intake on harbor seal serum FAA profiles were evaluated for two important representative prey fish diets, Pacific herring (*Clupea pallasi*) and walleye pollock (*Theragra chalcogramma*). Serum FAA and protein hydrolyzable amino acid compositions (HAA) in serum and red blood cells (RBCs) were also measured in several wild phocid species from Alaska, North Atlantic and Antarctica to provide baseline data on their amino acid composition.

MATERIALS AND METHODS

Blood samples from the captive harbor seals were routinely taken at two-week intervals after an overnight fast throughout the controlled feeding trial. Blood samples from wild harbor seals were collected in Prince William Sound and southeast Alaska in 1994 and archived by freezing at –80°C until analyzed. Blood samples from Atlantic

harbor seals were collected during a March 2000 field study located in the coastal waters of Maine. Blood samples from Weddell, Ross and crabeater seals were collected in the Eastern Ross Sea during a December 1999 through January 2000 field study in the Antarctica. In all cases, serum and RBCs were separated immediately after the blood collection and frozen at –80°C. Care was taken to avoid contamination of the serum with leukocytes. Serum samples were thawed and the protein precipitated before free amino acid analysis. Serum and RBC samples were freeze-dried, powdered for homogeneity, and hydrolyzed for protein amino acid analysis. Refer to Controlled Feeding Trial and Amino Acid Analysis Protocols in Chapter 2 for the details of the method.

RESULTS

HAA profiles in serum and RBC proteins of captive harbor seals

The mean HAA concentrations (μmol/g, dry weight) and mole percent compositions (%) in serum and RBC protein hydrolyzates from three captive harbor seals are reported in Table 6.1. HAA volume concentrations (μmol/l) in serum were obtained by a conversion based on an experimental linear relationship between serum dry weight and volume as shown in Fig. 6.1 (r^2=0.9993, n = 4). However, no such relationship was found for RBCs. Generally, HAA mole percent compositions in serum or RBCs were similar in all three captive harbor seals. HAA profiles in both serum and RBC protein hydrolyzates in the harbor seal Pender were fairly constant over time, as shown by repeated measurements over more than one year. These results indicated that HAA compositions were independent of either endogenous changes in harbor seal physiology,

Table 6.1a The mean ± SD of HAA concentrations (μmol/g, dry weight) in serum and RBC protein hydrolyzates of the three captive harbor seals

	Snapper serum		Poco serum		Pender serum		Snapper RBCs		Poco RBCs		Pender RBCs	
	Mean n=10	SD	Mean n=9	SD	Mean n=20	SD	Mean n=9	SD	Mean n=8	SD	Mean n=22	SD
Ala	535	73	553	31	463	19	876	53	937	45	799	58
Gly	308	43	308	22	253	16	546	49	569	49	483	41
Ser	230	62	228	15	163	27	196	40	217	58	170	66
Asx	496	83	513	33	435	20	694	79	716	105	677	51
Glx	680	128	739	62	670	35	412	45	416	49	413	32
Arg	241	35	249	13	208	11	166	21	168	11	397	33
His	128	20	137	8	113	6	454	70	484	42	142	13
Ile	136	20	130	13	143	27	87	12	109	38	81	10
Leu	570	82	595	35	513	26	896	60	954	57	831	62
Val	493	65	483	31	407	22	740	48	786	41	696	55
Lys	479	74	506	39	376	19	681	46	706	67	552	48
Phe	268	40	274	17	233	10	446	36	457	32	409	32
Tyr	195	29	185	8	153	10	175	13	149	40	124	23
Thr	303	53	291	14	235	17	351	35	366	49	315	49
BCAA[1]	1199	164	1208	77	1063	65	1723	102	1849	88	1608	122
EAA[2]	2813	410	2850	164	2382	123	3995	264	4178	272	3547	273
NEAA[3]	2248	376	2341	152	1984	98	2724	197	2855	276	2542	193
THAA[4]	5062	782	5191	314	4365	220	6719	456	7033	543	6089	463

Table 6.1b The mean ± SD of HAA mole percent compositions (%) in serum and RBC protein hydrolyzates of the three captive harbor seals

	Snapper serum		Poco serum		Pender serum		Snapper RBCs		Poco RBCs		Pender RBCs	
	Mean n=10	SD	Mean n=9	SD	Mean n=20	SD	Mean n=9	SD	Mean n=8	SD	Mean n=22	SD
Ala	10.6	0.6	10.7	0.2	10.6	0.2	13.0	0.5	13.4	0.6	13.1	0.4
Gly	6.1	0.1	5.9	0.1	5.8	0.2	8.1	0.4	8.1	0.2	7.9	0.2
Ser	4.4	0.8	4.4	0.2	3.7	0.5	2.9	0.5	3.0	0.6	2.8	1.0
Asx	9.8	0.3	9.9	0.2	10.0	0.3	10.3	0.7	10.1	0.9	11.1	0.3
Glx	13.3	0.6	14.2	0.5	15.3	0.1	6.2	0.9	5.9	0.4	6.8	0.3
Arg	4.8	0.1	4.8	0.1	4.8	0.1	2.5	0.4	2.4	0.2	2.3	0.1
His	2.5	0.1	2.6	0.1	2.6	0.1	6.7	0.8	6.9	0.3	6.5	0.2
Ile	2.7	0.2	2.5	0.1	3.3	0.5	1.3	0.3	1.6	0.7	1.3	0.1
Leu	11.3	0.3	11.5	0.1	11.8	0.1	13.3	0.4	13.6	0.4	13.7	0.3
Val	9.8	0.4	9.3	0.2	9.3	0.2	11.0	0.2	11.2	0.4	11.4	0.4
Lys	9.5	0.3	9.7	0.4	8.6	0.3	10.1	0.4	10.0	0.4	9.1	0.3
Phe	5.3	0.1	5.3	0.1	5.3	0.2	6.6	0.2	6.5	0.2	6.7	0.2
Tyr	3.9	0.1	3.6	0.2	3.5	0.2	2.6	0.3	2.1	0.5	2.0	0.3
Thr	5.9	0.2	5.6	0.1	5.4	0.2	5.2	0.3	5.2	0.4	5.2	0.6
BCAA[1]	23.8	0.8	23.3	0.4	24.3	0.5	25.7	0.5	26.4	1.4	26.4	0.7
EAA[2]	55.7	0.9	54.9	0.4	54.6	0.3	59.5	0.5	59.5	1.0	58.2	0.4
NEAA[3]	44.3	0.9	45.1	0.4	45.4	0.3	40.5	0.5	40.5	1.0	41.8	0.4
THAA[4]	100		100		100		100		100		100	

1. BCAA, branched-chain AA, including Val, Leu and Ile

2. EAA, including Arg, His, Ile, Leu, Val, Lys, Phe+Tyr and Thr

3. NEAA, including Ala, Gly, Ser, Asx (Asn+Asp) and Glx (Gln+Glu)

4. THAA, total hydrolyzed AA, not including Met

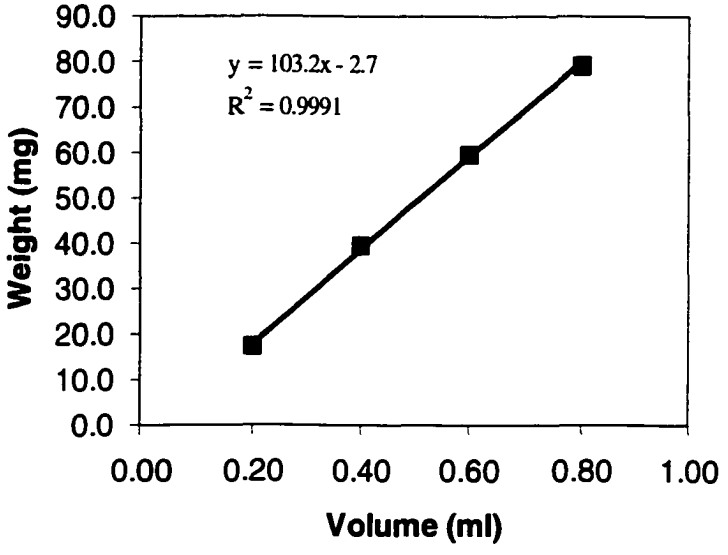


Fig. 6.1 A calibration line between serum dry weight and its volume.

such as molting or fasting, or exogenous factors such as dietary protein source or intake. HAA profiles reflected amino acid compositions of constituent proteins that were essentially constant and such amino acid compositions are unlikely to be useful for tracking short term variations in protein metabolism.

As shown in Table 6.1, concentrations of THAA in RBC proteins were almost double those in serum proteins, because of the high levels of hemoglobin in RBCs of harbor seals. Fig. 6.2 compares the HAA profiles of serum and RBCs in the harbor seal Pender. The HAA composition was Glx > Leu > Val > Ala > Asx > Lys > Gly > Thr > Phe > Ser > Arg > Ile > Tyr >His for serum proteins and Ala > Leu > Val > Asx > Lys > Gly > Glx > Phe > His > Thr > Ser > Arg > Tyr > Ile for RBC proteins. Significant differences in Glx and His levels were observed between serum and RBCs, with a high level of Glx and a relatively low level of His in serum compared with RBCs.

HAA profiles in serum proteins of wild phocids

HAA compositions of serum proteins were also measured in several wild seal species: harbor seals from Alaskan waters, harbor seals from the North Atlantic, Weddell, Ross and crabeater seals from the Antarctic Ocean. The mean amino acid compositions for these wild seal species are summarized in Table 6.2. HAA profiles were found to be indistinguishable among the four seal species.

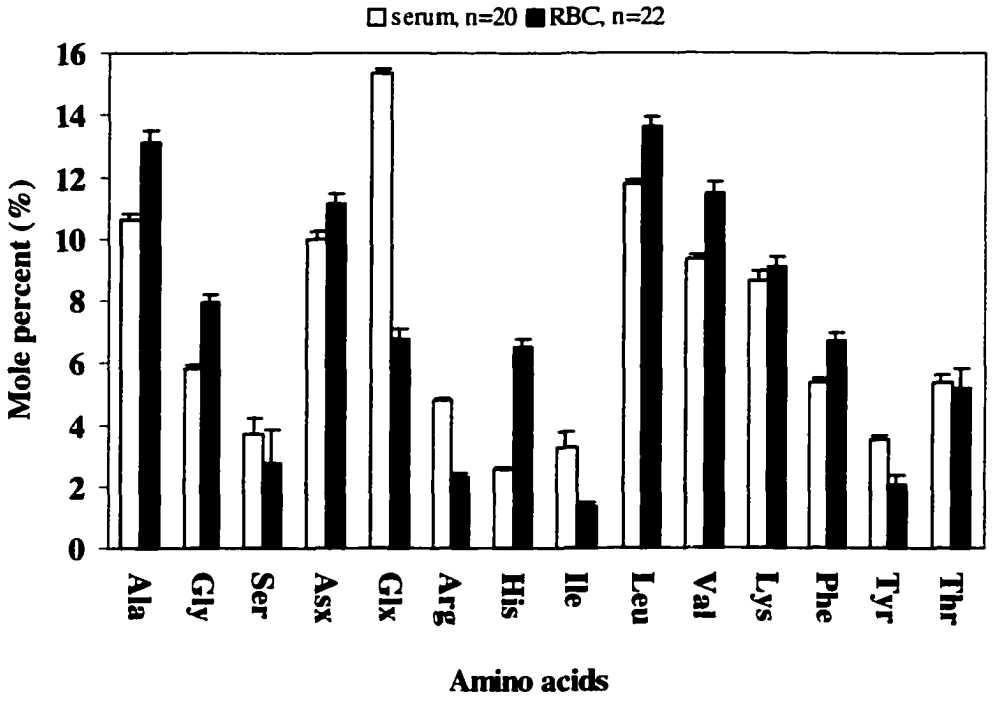


Fig. 6.2 Comparison of hydrolyzable amino acid composition between serum and RBC proteins in captive harbor seals.

Table 6.2a Comparison of HAA concentrations (μmol/g, dry weight) (mean ± SD) in serum protein hydrolyzates among wild seal species

	Harbor, Alaska		Harbor, Atlantic		Weddell		Ross		Crabeater	
	Mean n=4	SD	Mean n=5	SD	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD
Ala	462	66	485	36	487	23	520	15	495	13
Gly	372	98	366	100	361	38	310	28	303	25
Ser	262	40	243	41	286	48	242	32	228	48
Asx	423	52	447	39	473	31	482	37	470	41
Glx	677	85	693	59	739	34	775	31	734	22
Arg	215	28	215	23	213	8	227	8	219	1
His	120	15	132	11	134	12	128	7	124	5
Ile	177	24	168	16	193	16	177	23	163	6
Leu	538	74	559	42	560	26	571	26	559	8
Val	463	52	480	47	559	54	488	49	438	11
Lys	413	86	437	75	425	17	440	14	445	5
Phe	266	34	272	36	289	14	289	10	278	14
Tyr	166	16	156	20	184	19	174	9	169	4
Thr	296	32	287	30	319	35	286	31	263	14
BCAA[1]	1178	132	1207	93	1313	86	1237	96	1159	6
EAA[2]	2653	300	2706	239	2876	180	2781	167	2657	9
NEAA[3]	2196	297	2234	217	2347	152	2329	129	2229	57
THAA[4]	4848	588	4941	449	5223	332	5110	293	4885	52

Table 6.2b Comparison of HAA mole percent compositions (%) (mean ± SD) in serum protein hydrolyzates among wild seal species

	Harbor, Alaska		Harbor, Atlantic		Weddell		Ross		Crabeater	
	Mean n=4	SD	Mean n=5	SD	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD
Ala	9.5	0.4	9.8	0.2	9.3	0.5	10.2	0.3	10.1	0.3
Gly	7.6	1.4	7.4	1.6	6.9	0.3	6.1	0.2	6.2	0.4
Ser	5.4	0.4	4.9	0.5	5.4	0.6	4.7	0.4	4.7	0.9
Asx	8.7	0.2	9.1	0.4	9.1	0.1	9.4	0.6	9.6	0.8
Glx	14.0	0.7	14.1	0.8	14.2	0.5	15.2	0.3	15.0	0.6
Arg	4.4	0.1	4.4	0.1	4.1	0.1	4.4	0.1	4.5	0.1
His	2.5	0.1	2.7	0.2	2.6	0.1	2.5	0.0	2.5	0.1
Ile	3.7	0.4	3.4	0.3	3.7	0.1	3.5	0.3	3.3	0.1
Leu	11.1	0.3	11.3	0.2	10.7	0.5	11.2	0.2	11.4	0.3
Val	9.6	0.8	9.7	0.6	10.7	0.4	9.5	0.5	9.0	0.1
Lys	8.5	1.0	8.8	1.0	8.1	0.3	8.6	0.2	9.1	0.1
Phe	5.5	0.6	5.5	0.7	5.5	0.1	5.7	0.1	5.7	0.3
Tyr	3.4	0.4	3.1	0.2	3.5	0.3	3.4	0.2	3.5	0.1
Thr	6.1	0.3	5.8	0.1	6.1	0.4	5.6	0.3	5.4	0.2
BCAA[1]	24.3	1.0	24.5	0.9	25.1	0.1	24.2	0.7	23.7	0.3
EAA[2]	54.8	1.1	54.8	0.8	55.1	0.1	54.4	0.4	54.4	0.7
NEAA[3]	45.2	1.1	45.2	0.8	44.9	0.1	45.6	0.4	45.6	0.7
THAA[4]	100		100		100		100		100	

1. BCAA, branched-chain AA, including Val, Leu and Ile

2. EAA, including Arg, His, Ile, Leu, Val, Lys, Phe+Tyr and Thr

3. NEAA, including Ala, Gly, Ser, Asx (Asn+Asp) and Glx (Gln+Glu)

4. THAA, total hydrolyzed AA, not including Met

HAA profiles in Pacific herring and walleye pollock

The mean amino acid compositions of Pacific herring and walleye pollock are summarized in Table 6.3. The data revealed that AA mole percent compositions were similar between the two fish species (Fig. 6.3), although the THAA concentrations (μmol/g dry weight) in walleye pollock were approximately 1.5 times higher than those in Pacific herring, due to a much higher lipid content in the latter. Significant levels of taurine in fish tissue protein hydrolyzates were observed in both fish species.

Variations of serum FAA in captive seals in response to diets

The effects of dietary change on the fasting serum FAA compositions in captive harbor seals Travis and Pender were monitored over the course of a controlled feeding trial (Table 6.4). Glutamine was found to be the most abundant amino acid in serum, but it co-eluted with histidine. Their concentrations, reported as Gln+His, were calculated from the calibration curve of glutamine because free histidine concentrations in serum were much lower and less variable than those of glutamine (Armstrong and Stave II 1973). Alanine, an important component of the glucose-alanine cycle of muscle metabolism, ranked second among serum FAA (Fig. 6.4).

Variations of several serum free essential amino acids in response to diet switching were found in harbor seals Travis and Pender (Fig. 6.5). The mole percentages of essential branched-chain amino acids leucine, isoleucine and valine increased when the diet was switched from walleye pollock to Pacific herring, but decreased switching back

Table 6.3 Comparison of HAA concentrations (μmol/g, dry weight) and mole percent compositions (%) (mean ± SD) in tissue protein hydrolyzates of Pacific herring and walleye pollock.

	Concentrations (μmol/g dry weight)				Mole Percent Composition (%)			
	Pollock		Herring		Pollock		Herring	
	Mean n=8	SD	Mean n=10	SD	Mean n=8	SD	Mean n=10	SD
Ala	457	33	296	36	10.4	0.2	10.7	0.1
Gly	556	65	319	38	12.8	1.8	11.6	0.9
Ser	204	26	120	15	4.6	0.4	4.3	0.1
Asx	436	41	261	41	9.9	0.2	9.4	0.7
Glx	629	65	349	54	14.3	0.3	12.6	0.6
Arg	239	18	164	20	5.5	0.1	6.0	0.9
His	86	8	63	12	2.0	0.1	2.3	0.3
Ile	267	26	174	26	6.1	0.4	6.3	0.4
Leu	386	42	257	32	8.8	0.3	9.3	0.3
Val	308	34	218	27	7.0	0.3	7.9	0.2
Lys	343	40	217	32	7.8	0.3	7.8	0.3
Phe	176	30	123	16	4.0	0.4	4.5	0.3
Tyr	85	17	54	11	1.9	0.3	2.0	0.3
Thr	218	25	150	17	5.0	0.2	5.4	0.2
BCAA[1]	961	97	649	83	21.9	0.8	23.5	0.7
EAA[2]	2107	224	1420	166	47.9	1.5	51.4	1.0
NEAA[3]	2282	162	1345	173	52.1	1.5	48.6	1.0
THAA[4]	4390	369	2765	336	100		100	

1. BCAA, branched-chain AA, including Val, Leu and Ile

2. EAA, including Arg, His, Ile, Leu, Val, Lys, Phe+Tyr and Thr

3. NEAA, including Ala, Gly, Ser, Asx (Asn+Asp) and Glx (Gln+Glu)

4. THAA, total hydrolyzed AA, not including Met

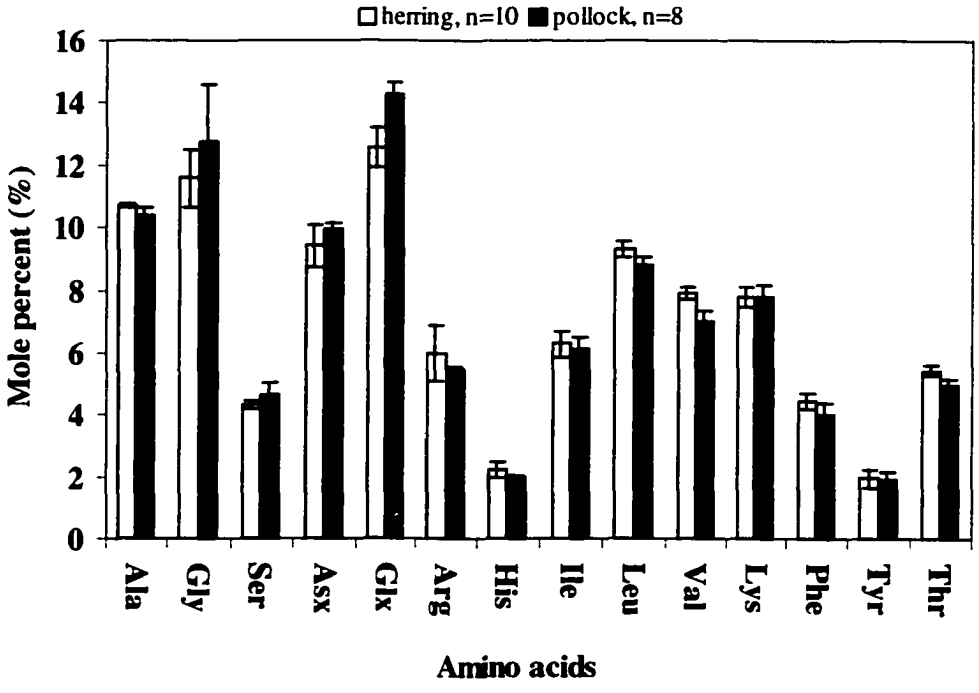


Fig. 6.3 Comparison of hydrolyzable amino acid compositions between Pacific herring and walleye pollock tissue proteins.

Table 6.4a Variations of serum FAA concentrations (μmol/l) in Travis over the course of controlled feeding trial

Sampling date	04/30/98	06/16/98	07/22/98	08/25/98	09/09/98	10/07/98	10/28/98	12/01/98	12/21/98	12/30/98
Ala	254	143	504	366	227	236	226	437	402	228
Gly	112	70	133	125	109	101	93	206	209	126
Ser	84	64	92	105	80	111	99	93	124	111
Asp	7	12	11	13	10	9	7	9	7	5
Asn	18	16	29	26	21	28	22	21	26	24
Glu	40	23	37	33	31	28	26	25	28	30
Gln+His	996	771	1026	949	867	1015	895	887	777	1126
Arg	115	77	113	119	97	108	87	129	187	101
Ile	42	38	48	65	52	64	38	50	75	77
Leu	78	83	86	132	106	165	91	81	116	145
Val	150	146	155	228	167	231	141	186	217	189
Lys	87	60	109	120	86	116	74	145	185	126
Phe	38	43	51	49	45	56	34	39	46	48
Tyr	33	20	41	38	29	35	25	48	39	29
Thr	105	64	97	105	94	93	78	139	117	89
Met	66	60	78	82	66	61	39	77	75	63
Tau	148	96	158	140	110	85	105	169	180	116
a-ABA	72	60	70	54	53	56	77	70	71	77
Orn	17	14	26	19	15	16	18	29	33	17
BCAA[1]	269	267	288	425	325	459	270	317	408	411
FEAA[2]	714	591	778	939	741	927	606	893	1057	867
FNEAA[3]	1511	1101	1832	1617	1345	1529	1369	1679	1573	1651
TFAA[4]	2462	1862	2864	2768	2263	2612	2175	2840	2914	2730
FAA Pool[5]	2225	1692	2611	2555	2086	2456	1975	2572	2630	2519

Table 6.4a continued (1)

02/09/99	02/16/99	03/02/99	03/09/99	03/25/99	04/06/99	04/20/99	05/05/99	05/19/99	06/01/99	06/15/99
281	161	198	353	385	199	197	224	299	270	225
154	110	116	112	121	85	80	97	136	154	149
129	82	92	96	104	92	75	84	99	105	98
12	12	12	11	11	12	10	9	10	11	10
31	21	23	21	28	19	18	19	21	28	25
37	33	28	31	34	31	26	43	33	30	35
1082	906	856	815	1069	945	803	979	1004	995	1196
127	79	84	90	99	73	71	86	94	159	129
89	45	49	74	63	78	41	45	41	56	72
201	96	106	155	143	177	97	106	91	117	153
296	142	152	223	217	239	134	154	150	191	250
156	74	106	102	129	81	64	68	91	112	104
66	47	47	53	63	49	45	46	51	56	71
46	28	34	41	38	29	24	26	37	50	32
128	76	94	89	94	68	57	72	99	104	118
82	63	75	84	77	62	55	53	70	72	77
122	132	84	124	125	93	92	97	146	137	149
0	23	13	7	77	68	65	68	66	82	80
20	18	17	17	20	16	14	13	20	20	19
586	283	308	452	423	495	272	305	281	364	475
1191	650	747	911	923	856	588	655	724	917	1006
1727	1326	1326	1439	1753	1383	1208	1457	1602	1593	1739
3059	2149	2187	2499	2898	2417	1968	2290	2558	2750	2992
2917	1976	2073	2350	2676	2239	1797	2112	2326	2510	2744

Table 6.4a continued (2)

06/29/99	07/13/99	07/27/99	08/10/99	08/25/99	09/07/99	09/16/99	10/05/99	10/19/99	11/02/99	11/16/99
231	191	218	257	205	141	213	295	173	109	250
147	123	170	163	171	121	184	184	137	71	103
109	95	109	107	147	85	114	131	82	52	87
9	12	11	11	10	9	10	11	16	10	11
24	24	28	28	34	26	33	34	26	15	25
41	40	46	40	40	31	37	40	28	15	24
1136	983	1285	1143	1273	952	1164	1260	997	553	826
131	122	151	112	143	102	125	150	101	50	82
63	75	100	77	102	81	102	91	72	40	53
139	151	160	124	191	157	199	181	144	85	104
216	213	229	205	310	260	313	298	223	131	154
103	97	142	139	170	105	135	140	101	55	97
51	53	52	61	65	57	71	74	53	30	48
36	31	39	40	41	32	44	43	30	18	32
118	97	113	78	142	107	139	144	98	52	81
70	66	76	82	88	75	99	92	70	40	61
124	121	176	183	110	93	146	151	99	41	88
79	78	83	93	80	69	83	76	0	8	14
18	19	20	17	25	19	28	25	18	13	15
418	439	489	407	603	498	614	570	440	256	311
926	904	1060	919	1251	976	1228	1213	892	500	711
1697	1467	1866	1750	1880	1364	1755	1956	1459	826	1325
2844	2589	3205	2963	3345	2521	3239	3420	2468	1388	2153
2623	2371	2926	2669	3131	2339	2983	3168	2351	1326	2036

Table 6.4a continued (3)

12/03/99	12/14/99	12/28/99	01/11/00	02/01/00	02/15/00	02/29/00	03/14/00	03/28/00	04/11/00	04/25/00
167	164	230	327	267	129	611	194	209	280	327
93	88	108	109	133	97	168	147	134	187	143
71	77	83	71	84	68	91	81	71	113	114
12	12	8	11	10	11	10	9	10	13	5
20	17	23	22	23	18	31	21	17	30	31
23	30	25	31	24	29	36	28	36	154	35
770	781	816	820	854	833	1182	1081	932	910	1175
67	66	74	71	88	64	127	104	98	114	112
38	44	38	34	50	46	55	55	58	75	80
76	102	71	64	102	98	94	101	102	143	154
119	140	108	98	159	146	170	179	182	238	230
81	63	84	81	87	53	116	96	86	130	133
42	41	41	44	43	34	50	38	39	69	62
30	31	31	33	25	20	37	25	21	31	40
67	63	71	76	87	72	130	98	87	134	119
55	57	55	60	49	36	70	55	52	77	86
80	73	90	87	98	87	209	154	128	146	139
6	6	8	5	16	25	19	20	20	67	67
16	16	15	18	17	14	22	18	18	20	21
233	287	217	195	312	290	319	335	342	455	464
574	607	573	560	691	570	848	752	726	1011	1017
1156	1168	1293	1392	1395	1185	2130	1561	1409	1687	1831
1832	1870	1979	2061	2217	1881	3228	2506	2300	2930	3074
1730	1775	1866	1951	2086	1755	2979	2314	2135	2698	2847

Table 6.4a continued (4)

05/12/00	05/25/00	06/13/00	07/11/00	07/25/00	08/12/00	08/22/00
267	295	162	317	365	259	291
165	197	94	145	150	141	124
108	131	77	126	133	98	121
10	6	4	5	8	8	9
30	40	20	36	35	28	30
45	32	18	32	53	31	31
1215	1414	913	1204	1161	1073	1096
135	142	84	115	108	100	119
82	108	55	68	71	45	68
136	230	122	151	146	87	140
227	323	183	217	225	153	224
126	156	76	133	131	94	109
57	77	52	70	69	55	60
38	49	26	50	48	48	47
129	158	81	110	122	98	104
83	99	66	89	91	68	79
142	180	89	119	164	140	158
68	79	66	69	63	63	76
24	25	15	21	26	19	16
444	661	360	436	442	285	432
1013	1342	743	1003	1012	749	950
1840	2116	1288	1865	1906	1639	1702
3088	3742	2202	3077	3171	2611	2902
2852	3458	2031	2868	2917	2389	2652

Table 6.4b Variations of serum FAA mole percent compositions (%) in Travis over the course of controlled feeding tria

Sampling date	04/30/98	06/16/98	07/22/98	08/25/98	09/09/98	10/07/98	10/28/98	12/01/98	12/21/98	12/30/98
Ala	11.4	8.5	19.3	14.3	10.9	9.6	11.4	17.0	15.3	9.1
Gly	5.0	4.2	5.1	4.9	5.2	4.1	4.7	8.0	8.0	5.0
Ser	3.8	3.8	3.5	4.1	3.9	4.5	5.0	3.6	4.7	4.4
Asp	0.3	0.7	0.4	0.5	0.5	0.4	0.4	0.4	0.2	0.2
Asn	0.8	1.0	1.1	1.0	1.0	1.1	1.1	0.8	1.0	1.0
Glu	1.8	1.4	1.4	1.3	1.5	1.2	1.3	1.0	1.1	1.2
Gln+His	44.8	45.6	39.3	37.1	41.6	41.3	45.3	34.5	29.5	44.7
Arg	5.2	4.5	4.3	4.6	4.7	4.4	4.4	5.0	7.1	4.0
Ile	1.9	2.2	1.8	2.6	2.5	2.6	1.9	1.9	2.8	3.1
Leu	3.5	4.9	3.3	5.2	5.1	6.7	4.6	3.2	4.4	5.7
Val	6.7	8.7	5.9	8.9	8.0	9.4	7.1	7.2	8.3	7.5
Lys	3.9	3.5	4.2	4.7	4.1	4.7	3.7	5.6	7.0	5.0
Phe	1.7	2.5	2.0	1.9	2.1	2.3	1.7	1.5	1.7	1.9
Tyr	1.5	1.2	1.6	1.5	1.4	1.4	1.3	1.9	1.5	1.2
Thr	4.7	3.8	3.7	4.1	4.5	3.8	3.9	5.4	4.5	3.5
Met	3.0	3.5	3.0	3.2	3.2	2.5	2.0	3.0	2.9	2.5
BCAA[1]	12.1	15.8	11.0	16.6	15.6	18.7	13.7	12.3	15.5	16.3
FEAA[2]	32.1	34.9	29.8	36.7	35.5	37.8	30.7	34.7	40.2	34.4
FNEAA[3]	67.9	65.1	70.2	63.3	64.5	62.2	69.3	65.3	59.8	65.6
FAA Pool[5]	100	100	100	100	100	100	100	100	100	100

1. BCAA, branched-chain AA, including Val, Leu and Ile.

2. FEAA, including Arg, His+Gln, Iie, Leu, Val, Lys, Phe+Tyr and Thr.

3. FNEAA, including Ala, Gly, Ser, Asx (Asn+Asp) and Glx (Gln+Glu).

4. TFAA, total free AA.

5. FAA Pool, including total free AA except taurine (Tau), α-amino butyric acid (α-ABA) and ornithine (Orn).

Table 6.4b continued (1)

02/09/99	02/16/99	03/02/99	03/09/99	03/25/99	04/06/99	04/20/99	05/05/99	05/19/99	06/01/99	06/15/99
9.6	8.2	9.6	15.0	14.4	8.9	11.0	10.6	12.8	10.8	8.2
5.3	5.6	5.6	4.8	4.5	3.8	4.4	4.6	5.8	6.1	5.4
4.4	4.2	4.5	4.1	3.9	4.1	4.2	4.0	4.2	4.2	3.6
0.4	0.6	0.6	0.5	0.4	0.5	0.5	0.4	0.4	0.4	0.4
1.1	1.0	1.1	0.9	1.0	0.8	1.0	0.9	0.9	1.1	0.9
1.3	1.7	1.4	1.3	1.3	1.4	1.4	2.0	1.4	1.2	1.3
37.1	45.9	41.3	34.7	40.0	42.2	44.7	46.4	43.2	39.6	43.6
4.4	4.0	4.0	3.8	3.7	3.3	3.9	4.1	4.1	6.3	4.7
3.0	2.3	2.4	3.2	2.3	3.5	2.3	2.1	1.7	2.2	2.6
6.9	4.8	5.1	6.6	5.3	7.9	5.4	5.0	3.9	4.7	5.6
10.1	7.2	7.3	9.5	8.1	10.7	7.5	7.3	6.4	7.6	9.1
5.3	3.8	5.1	4.3	4.8	3.6	3.6	3.2	3.9	4.5	3.8
2.3	2.4	2.3	2.3	2.4	2.2	2.5	2.2	2.2	2.2	2.6
1.6	1.4	1.6	1.8	1.4	1.3	1.3	1.2	1.6	2.0	1.2
4.4	3.8	4.5	3.8	3.5	3.0	3.2	3.4	4.3	4.2	4.3
2.8	3.2	3.6	3.6	2.9	2.8	3.1	2.5	3.0	2.9	2.8
20.1	14.3	14.8	19.2	15.8	22.1	15.2	14.5	12.1	14.5	17.3
40.8	32.9	36.0	38.8	34.5	38.2	32.8	31.0	31.1	36.5	36.6
59.2	67.1	64.0	61.2	65.5	61.8	67.2	69.0	68.9	63.5	63.4
100	100	100	100	100	100	100	100	100	100	100

Table 6.4b continued (2)

06/29/99	07/13/99	07/27/99	08/10/99	08/25/99	09/07/99	09/16/99	10/05/99	10/19/99	11/02/99	11/16/99
8.8	8.1	7.4	9.6	6.5	6.0	7.1	9.3	7.4	8.2	12.3
5.6	5.2	5.8	6.1	5.5	5.2	6.2	5.8	5.8	5.4	5.1
4.1	4.0	3.7	4.0	4.7	3.6	3.8	4.1	3.5	4.0	4.3
0.3	0.5	0.4	0.4	0.3	0.4	0.3	0.4	0.7	0.7	0.5
0.9	1.0	0.9	1.1	1.1	1.1	1.1	1.1	1.1	1.2	1.2
1.6	1.7	1.6	1.5	1.3	1.3	1.2	1.3	1.2	1.2	1.2
43.3	41.4	43.9	42.8	40.7	40.7	39.0	39.8	42.4	41.7	40.6
5.0	5.1	5.1	4.2	4.6	4.4	4.2	4.7	4.3	3.7	4.0
2.4	3.2	3.4	2.9	3.3	3.5	3.4	2.9	3.1	3.0	2.6
5.3	6.4	5.5	4.7	6.1	6.7	6.7	5.7	6.1	6.4	5.1
8.2	9.0	7.8	7.7	9.9	11.1	10.5	9.4	9.5	9.9	7.5
3.9	4.1	4.8	5.2	5.4	4.5	4.5	4.4	4.3	4.2	4.8
1.9	2.2	1.8	2.3	2.1	2.4	2.4	2.3	2.2	2.2	2.3
1.4	1.3	1.3	1.5	1.3	1.4	1.5	1.4	1.3	1.4	1.6
4.5	4.1	3.9	2.9	4.5	4.6	4.7	4.5	4.1	3.9	4.0
2.7	2.8	2.6	3.1	2.8	3.2	3.3	2.9	3.0	3.0	3.0
15.9	18.5	16.7	15.3	19.3	21.3	20.6	18.0	18.7	19.3	15.3
35.3	38.1	36.2	34.4	40.0	41.7	41.2	38.3	38.0	37.7	34.9
64.7	61.9	63.8	65.6	60.0	58.3	58.8	61.7	62.0	62.3	65.1
100	100	100	100	100	100	100	100	100	100	100

Table 6.4b continued (3)

12/03/99	12/14/99	12/28/99	01/11/00	02/01/00	02/15/00	02/29/00	03/14/00	03/28/00	04/11/00	04/25/00
9.7	9.2	12.3	16.7	12.8	7.3	20.5	8.4	9.8	10.4	11.5
5.4	4.9	5.8	5.6	6.4	5.5	5.7	6.3	6.3	6.9	5.0
4.1	4.3	4.4	3.7	4.0	3.8	3.1	3.5	3.3	4.2	4.0
0.7	0.6	0.5	0.6	0.5	0.6	0.3	0.4	0.5	0.5	0.2
1.2	1.0	1.2	1.1	1.1	1.0	1.1	0.9	0.8	1.1	1.1
1.3	1.7	1.3	1.6	1.2	1.7	1.2	1.2	1.7	5.7	1.2
44.5	44.0	43.7	42.0	40.9	47.5	39.7	46.7	43.7	33.7	41.3
3.9	3.7	3.9	3.7	4.2	3.7	4.3	4.5	4.6	4.2	3.9
2.2	2.5	2.0	1.7	2.4	2.6	1.8	2.4	2.7	2.8	2.8
4.4	5.8	3.8	3.3	4.9	5.6	3.2	4.4	4.8	5.3	5.4
6.9	7.9	5.8	5.0	7.6	8.3	5.7	7.7	8.5	8.8	8.1
4.7	3.6	4.5	4.1	4.2	3.0	3.9	4.2	4.0	4.8	4.7
2.4	2.3	2.2	2.2	2.1	1.9	1.7	1.7	1.8	2.5	2.2
1.7	1.7	1.7	1.7	1.2	1.2	1.2	1.1	1.0	1.2	1.4
3.9	3.5	3.8	3.9	4.2	4.1	4.4	4.2	4.1	5.0	4.2
3.2	3.2	2.9	3.1	2.4	2.0	2.3	2.4	2.5	2.9	3.0
13.5	16.2	11.6	10.0	14.9	16.5	10.7	14.5	16.0	16.9	16.3
33.2	34.2	30.7	28.7	33.1	32.5	28.5	32.5	34.0	37.5	35.7
66.8	65.8	69.3	71.3	66.9	67.5	71.5	67.5	66.0	62.5	64.3
100	100	100	100	100	100	100	100	100	100	100

Table 6.4b continued (4)

05/12/00	05/25/00	06/13/00	07/11/00	07/25/00	08/12/00	08/22/00
9.4	8.5	8.0	11.1	12.5	10.8	11.0
5.8	5.7	4.6	5.1	5.2	5.9	4.7
3.8	3.8	3.8	4.4	4.6	4.1	4.6
0.3	0.2	0.2	0.2	0.3	0.3	0.3
1.0	1.2	1.0	1.3	1.2	1.2	1.1
1.6	0.9	0.9	1.1	1.8	1.3	1.2
42.6	40.9	44.9	42.0	39.8	44.9	41.3
4.7	4.1	4.1	4.0	3.7	4.2	4.5
2.9	3.1	2.7	2.4	2.4	1.9	2.6
4.8	6.6	6.0	5.3	5.0	3.6	5.3
7.9	9.3	9.0	7.6	7.7	6.4	8.4
4.4	4.5	3.7	4.6	4.5	3.9	4.1
2.0	2.2	2.5	2.4	2.4	2.3	2.3
1.3	1.4	1.3	1.7	1.7	2.0	1.8
4.5	4.6	4.0	3.8	4.2	4.1	3.9
2.9	2.9	3.2	3.1	3.1	2.9	3.0
15.6	19.1	17.7	15.2	15.1	11.9	16.3
35.5	38.8	36.6	35.0	34.7	31.4	35.8
64.5	61.2	63.4	65.0	65.3	68.6	64.2
100	100	100	100	100	100	100

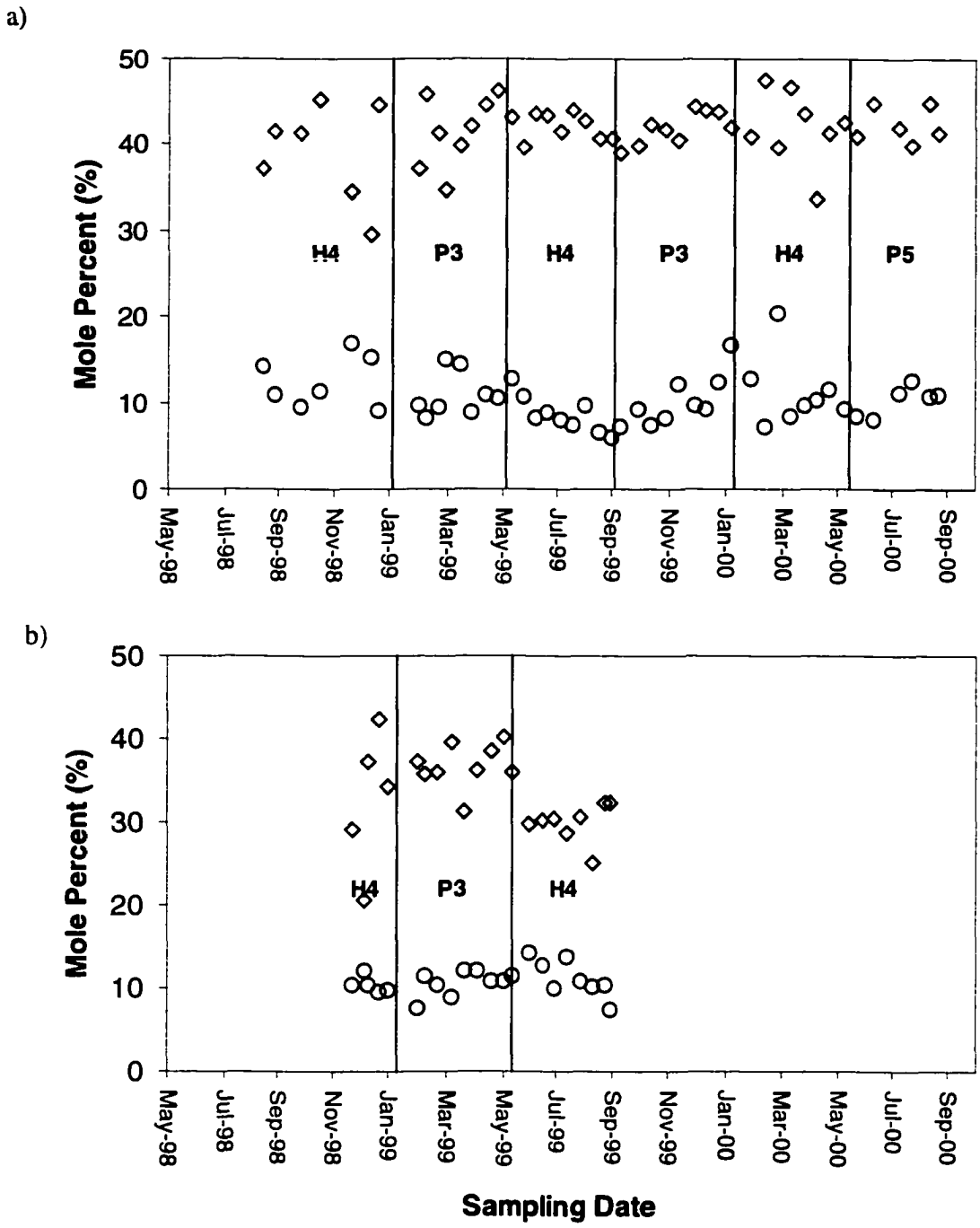


Fig. 6.4 Variations of serum free glutamine (plus histidine) and alanine composition in response to diet changes in captive harbor seals, a) Travis and b) Pender, over the course of the controlled feeding trial. ◇ Gln(+His); ○ Ala.

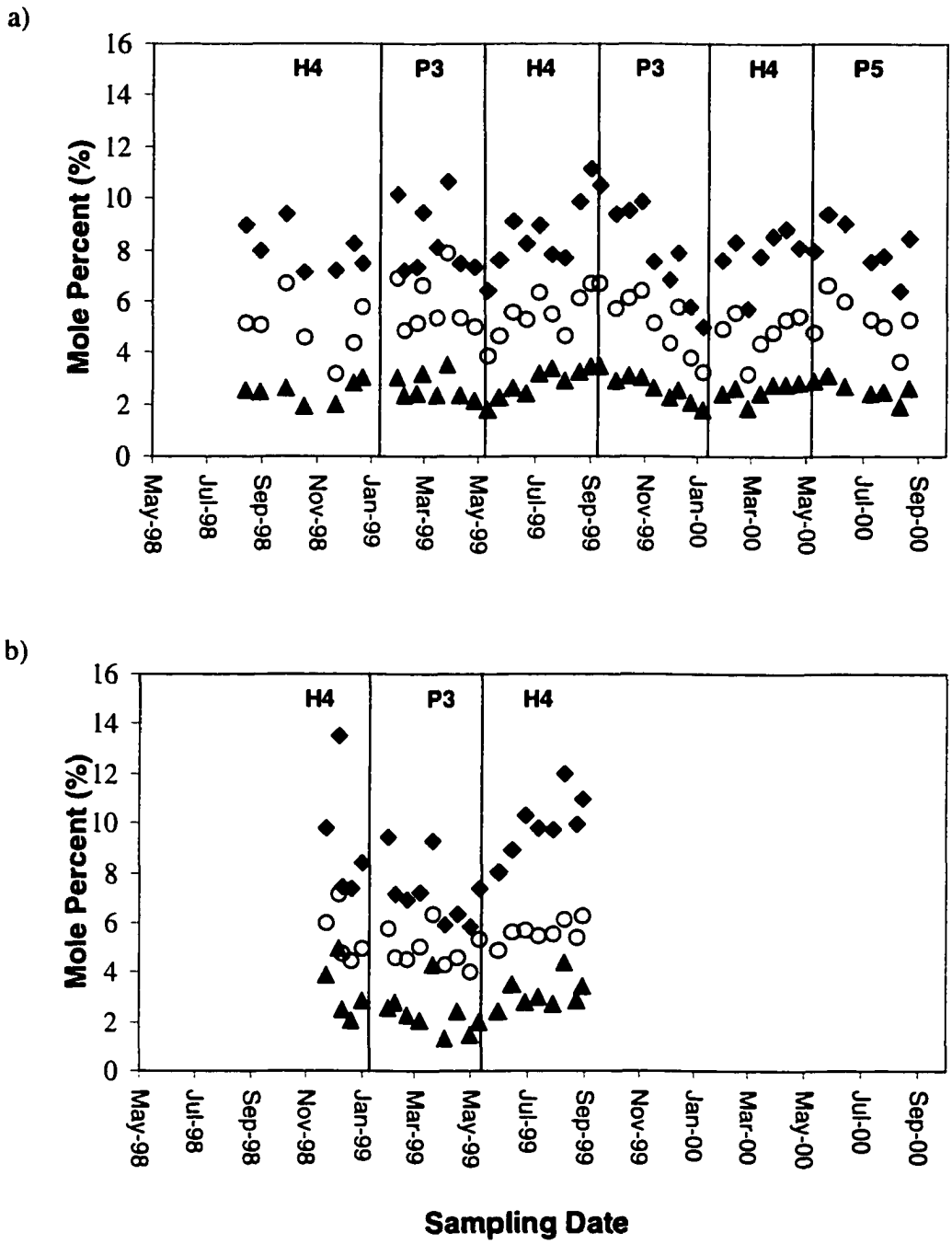
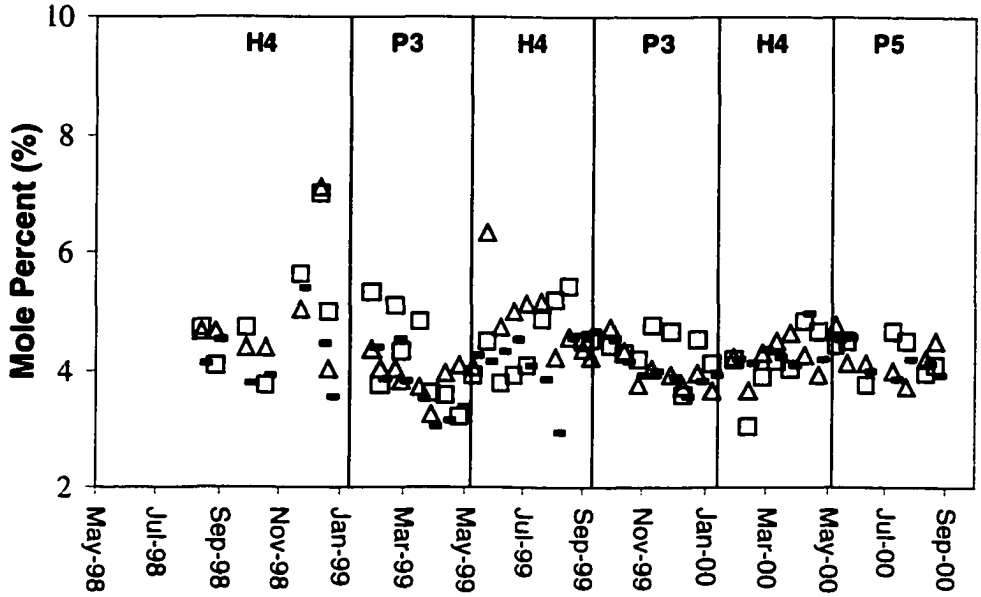


Fig. 6.5a, b Variations of several serum free essential amino acids in response to diet changes in captive harbor seals, Travis and Pender, over the course of the controlled feeding trial. a) ◆Val, ○Leu and ▲Ile in Travis; b) ◆Val, ○Leu and ▲Ile in Pender.

c)



d)

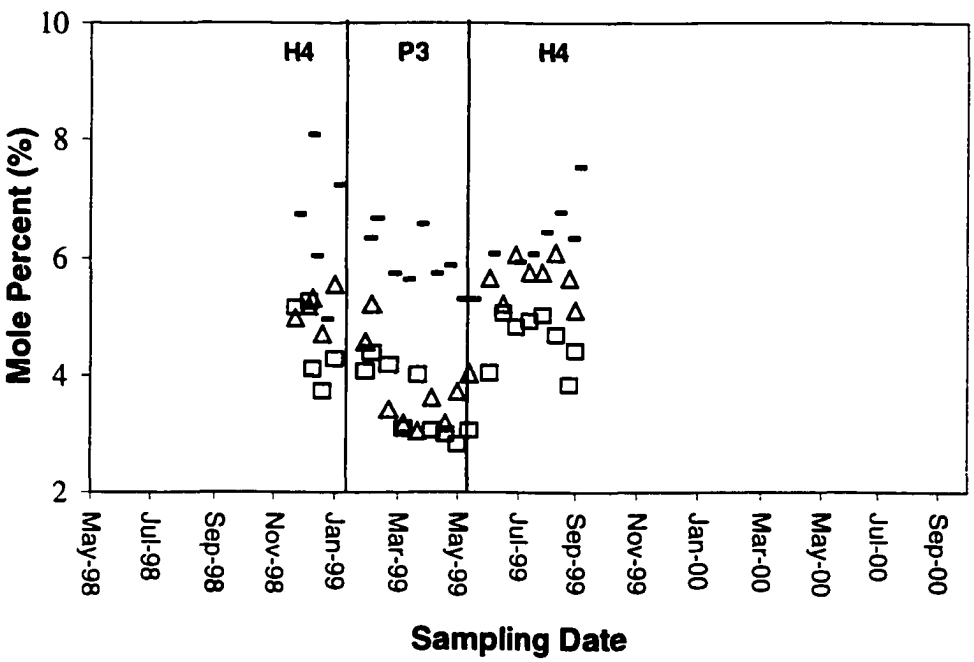


Fig. 6.5c, d Variations of several serum free essential amino acids in response to diet changes in captive harbor seals, Travis and Pender, over the course of the controlled feeding trial. c) □ Lys, △ Arg and ▪Thr in Travis; d) Lys, △ Arg and ▪Thr in Pender.

to walleye pollock. The free essential amino acids lysine, arginine and threonine also seemed to have a similar pattern, but to a lesser extent. In contrast to the above essential amino acids, alanine followed a reverse pattern. As shown in Fig. 6.4, the mole percentages of serum free alanine increased on the pollock diet and decreased upon switching to herring in some feeding cycles. When compared with the daily body protein intake of Travis and Pender in the different feeding cycles (Fig. 3.7), the above patterns suggested a correlation of serum FAA profiles and protein intake. Higher serum free EAA levels corresponded to the lower protein intake on a herring diet. In addition to the protein FAA, significant levels of free serum taurine, α-amino butyric acid and ornithine were observed in Travis and Pender (see Table 6.4).

Comparison of serum FAA profiles among wild phocids

Serum FAA compositions were compared among several wild seal species. Total free amino acid pool sizes were similar among all four species (Table 6.5). However, the relative proportions of individual amino acids varied slightly (Table 6.6). In particular, the mole percentage of valine in crabeater and Ross seals was generally lower than that in Weddell and Harbor seals (Fig.6.6a). The mole percentage of alanine also differed among seal species and mirrored the pattern of valine (Fig. 6.6b). The mole percentage of alanine was higher in crabeater and Ross seals than that in Weddell and harbor seals. Generally speaking, Weddell and harbor seals were most similar in their serum FAA compositions. Serum FAA compositions in crabeater seals were more similar to those in Ross seals, but significantly different from those in Weddell or harbor seals.

Table 6.5a Serum FAA concentrations (µmol/l) in wild seal species

Sample ID	W2597	W2598	W2600	W2667	W2701	R2705	R2706
Species	Weddell	Weddell	Weddell	Weddell	Weddell	Ross	Ross
Ala	437	285	622	319	486	263	362
Gly	217	209	210	189	206	134	171
Ser	141	161	149	130	135	100	101
Asn	52	60	55	41	43	46	52
Asp	N.D.*	N.D.	N.D.	N.D.	11	N.D.	N.D.
Glu	51	35	36	53	64	56	90
Arg	133	163	131	105	116	121	99
Gln+His	783	550	988	864	754	1216	796
Ile	95	119	111	79	104	66	76
Leu	141	143	200	201	195	106	129
Val	202	207	225	254	300	129	156
Lys	170	164	267	165	128	156	221
Phe	77	78	87	86	62	37	76
Tyr	51	34	60	46	54	44	54
Thr	221	218	241	103	121	133	152
Met	86	80	80	64	95	69	79
Tau	191	239	112	108	191	94	103
a-ABA	44	48	28	24	24	32	29
Orn	66	105	41	43	56	52	35
BCAA[1]	437	468	535	535	599	301	361
FEAA[2]	1958	1755	2390	1967	1927	2075	1837
FNEAA[3]	899	751	1072	733	945	599	776
TFAA[4]	3157	2898	3643	2876	3144	2852	2779
FAA Pool[5]	2857	2506	3462	2700	2872	2674	2613

Table 6.5a continued

R2736 Ross	R2742 Ross	R2862 Ross	C2716 Crabeater	C2747 Crabeater	C2756 Crabeater	C2761 Crabeater	C2766 Crabeater
857	664	696	787	616	633	796	627
233	355	188	201	221	164	160	187
135	197	101	162	116	104	125	144
53	61	49	78	53	59	55	75
N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
48	45	56	45	68	41	37	60
140	166	105	133	167	148	128	144
925	811	1021	859	766	757	623	977
83	96	80	65	68	73	51	137
101	132	131	118	123	125	94	185
128	176	154	173	149	184	131	194
214	168	120	188	127	166	122	285
61	62	59	64	56	57	44	68
92	64	54	81	56	63	62	64
131	133	157	204	101	140	115	163
105	133	91	89	67	78	64	112
85	312	102	166	337	175	187	198
51	31	29	61	33	49	51	55
58	99	80	58	36	40	68	75
312	404	364	356	341	383	276	516
1980	1942	1971	1973	1680	1791	1434	2331
1326	1322	1090	1272	1074	1001	1172	1093
3500	3706	3272	3530	3160	3057	2913	3751
3305	3265	3061	3244	2753	2793	2607	3423

Table 6.5b Serum FAA mole percent composition (%) based on FAA pool in wild seal species

Sample ID	W2597	W2598	W2600	W2667	W2701	R2705	R2706
Species	Weddell	Weddell	Weddell	Weddell	Weddell	Ross	Ross
Ala	15.3	11.4	18.0	11.8	16.9	9.8	13.8
Gly	7.6	8.4	6.1	7.0	7.2	5.0	6.6
Ser	4.9	6.4	4.3	4.8	4.7	3.7	3.9
Asp	N.D.	N.D.	N.D.	N.D.	0.4	N.D.	N.D.
Asn	1.8	2.4	1.6	1.5	1.5	1.7	2.0
Glu	1.8	1.4	1.1	1.9	2.2	2.1	3.4
Gln+His	27.4	21.9	28.5	32.0	26.2	45.5	30.5
Arg	4.6	6.5	3.8	3.9	4.0	4.5	3.8
Ile	3.3	4.7	3.2	2.9	3.6	2.5	2.9
Leu	4.9	5.7	5.8	7.4	6.8	4.0	4.9
Val	7.1	8.3	6.5	9.4	10.5	4.8	6.0
Lys	5.9	6.5	7.7	6.1	4.4	5.8	8.5
Phe	2.7	3.1	2.5	3.2	2.2	1.4	2.9
Tyr	1.8	1.4	1.7	1.7	1.9	1.6	2.0
Thr	7.7	8.7	7.0	3.8	4.2	5.0	5.8
Met	3.0	3.2	2.3	2.4	3.3	2.6	3.0
BCAA[1]	15.3	18.7	15.5	19.8	20.9	11.3	13.8
FEAA[2]	41.1	48.1	40.5	40.8	40.9	32.1	39.8
FNEAA[3]	58.9	51.9	59.5	59.2	59.1	67.9	60.2
FAA Pool[5]	100	100	100	100	100	100	100

1. BCAA, branched-chain AA, including Val, Leu and Iie.

2. FEAA, including Arg, His+Gln, Ile, Leu, Val, Lys, Phe+Tyr and Thr.

3. FNEAA, including Ala, Gly, Ser, Asx (Asn+Asp) and Glx (Gln+Glu).

4. TFAA, total free AA.

5. FAA Pool, including total free AA except taurine (Tau), α-amino butyric acid (α-ABA) and ornithine (Orn).

*** not detected**

Table 6.5b continued

R2736 Ross	R2742 Ross	R2862 Ross	C2716 Crabeater	C2747 Crabeater	C2756 Crabeater	C2761 Crabeater	C2766 Crabeater
25.9	20.4	22.8	24.2	22.4	22.7	30.5	18.3
7.1	10.9	6.2	6.2	8.0	5.9	6.1	5.5
4.1	6.0	3.3	5.0	4.2	3.7	4.8	4.2
N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1.6	1.9	1.6	2.4	1.9	2.1	2.1	2.2
1.4	1.4	1.8	1.4	2.5	1.5	1.4	1.8
28.0	24.9	33.4	26.5	27.8	27.1	23.9	28.5
4.2	5.1	3.4	4.1	6.1	5.3	4.9	4.2
2.5	2.9	2.6	2.0	2.5	2.6	1.9	4.0
3.0	4.0	4.3	3.6	4.5	4.5	3.6	5.4
3.9	5.4	5.0	5.3	5.4	6.6	5.0	5.7
6.5	5.2	3.9	5.8	4.6	5.9	4.7	8.3
1.9	1.9	1.9	2.0	2.0	2.0	1.7	2.0
2.8	2.0	1.7	2.5	2.0	2.2	2.4	1.9
4.0	4.1	5.1	6.3	3.7	5.0	4.4	4.8
3.2	4.1	3.0	2.7	2.4	2.8	2.4	3.3
9.4	12.4	11.9	11.0	12.4	13.7	10.6	15.1
31.9	34.6	31.0	34.3	33.2	37.0	31.1	39.5
68.1	65.4	69.0	65.7	66.8	63.0	68.9	60.5
100	100	100	100	100	100	100	100

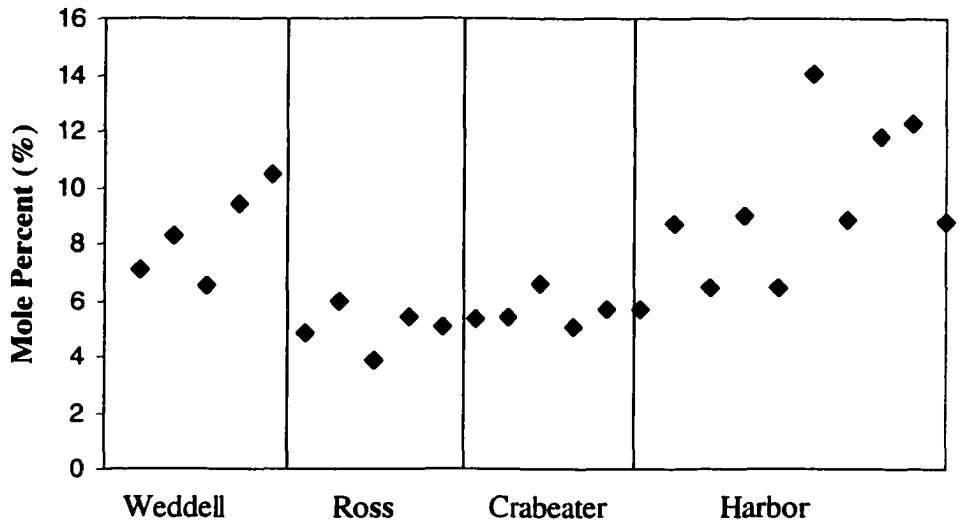
Table 6.6 Comparison of serum FAA mole percent compositions (%) (mean ± SD) based on TFAA among wild seal species

	Harbor, Alaska		Weddell		Ross		Crabeater	
	Mean n=12	SD	Mean n=5	SD	Mean n=5	SD	Mean n=5	SD
Ala	10.4	3.9	13.5	3.0	17.2	6.2	21.3	3.9
Gly	5.5	0.7	6.6	0.5	6.6	1.8	5.7	0.8
Ser	3.7	0.5	4.6	0.6	3.9	0.9	4.0	0.5
Asp	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0
Asn	1.0	0.2	1.6	0.3	1.6	0.2	1.9	0.2
Glu	2.0	0.6	1.5	0.4	1.9	0.8	1.5	0.4
Gln+His	31.3	4.5	25.0	4.1	30.2	7.8	24.2	1.7
Arg	3.8	1.6	4.1	0.9	3.9	0.5	4.4	0.6
Ile	2.8	0.9	3.2	0.5	2.5	0.2	2.4	0.8
Leu	4.9	1.6	5.6	1.0	3.8	0.6	3.9	0.7
Val	8.2	2.3	7.6	1.5	4.6	0.7	5.1	0.6
Lys	4.1	0.6	5.6	1.2	5.5	1.6	5.3	1.4
Phe	1.9	0.4	2.5	0.4	1.9	0.5	1.8	0.1
Tyr	1.8	0.7	1.5	0.2	1.9	0.4	2.0	0.2
Thr	5.0	0.6	5.7	1.9	4.5	0.8	4.4	1.0
Met	2.3	0.3	2.6	0.4	2.9	0.4	2.5	0.3
Tau	8.3	2.3	5.4	2.1	4.2	2.4	6.6	2.4
a-ABA	1.3	0.6	1.1	0.4	1.1	0.3	1.5	0.3
Orn	1.8	1.4	2.0	1.0	2.0	0.6	1.7	0.5
BCAA[1]	15.9	4.6	16.5	2.3	10.9	1.5	11.3	1.8
TFAA[2]	100.0		100.0		100.0		100.0	

1. BCAA, branched-chain AA, including Val, Leu and Ile

2. TFAA, total free AA

a)



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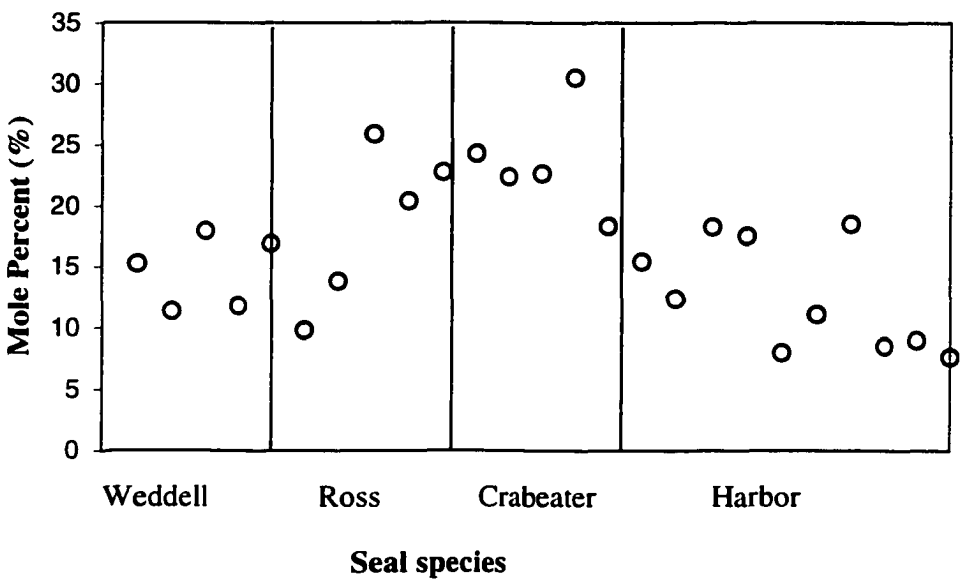


Fig. 6.6 Comparison of a) serum free valine (◆) and b) alanine (○) mole percent composition among four wild seal species.

DISCUSSION

AA compositions of protein hydrolyzates

As expected, amino acid profiles in both serum and RBC proteins are relatively uniform among different seal species. The differences in amino acid mole percent composition between serum and RBCs reflect the differing compositions of the constituent proteins. Distinct differences in amino acid composition between seals and pelagic fish reflect differences in the constituent proteins that comprise serum and RBCs and fish muscle. Relatively high taurine levels were found in fish tissue hydrolyzates of both Pacific herring and walleye pollock, which is consistent with the fact that usually relatively high concentration of nonessential amino acid taurine presents in muscle tissue (Ward et al. 1999).

Variations of serum FAA in captive seals in response to diets

The dietary effects on the fasting serum or plasma FAA compositions in harbor seals have not been investigated previously. Unlike hydrolyzable amino acids, serum FAA compositions varied significantly in response to the two different fish diets. In harbor seal Travis, glutamine (plus histidine) ranged from 34.3% to 48.3% and alanine from 6.1% to 17.2%. The considerable variations in serum free glutamine and alanine levels could be closely associated to their functions in regulation of protein metabolism. It is well known that all 20 protein amino acids are channeled into two major amino acids: alanine for transport of α-amino groups from skeletal muscle to liver, via the glucose-alanine cycle, and glutamine for disposal or storage of excess nitrogen via

transamination or oxidative deamination reactions (Lehninger et al. 1993). The levels of serum free glutamine and alanine may be controlled by liver metabolism, which in turn is governed by the balance between endogenous amino acid requirements and exogenous protein intake. As shown in Fig. 6.4, the elevated serum free alanine levels in Travis and Pender were significant when the diet was switched from herring to pollock. Since alanine serves as a carrier in transporting ammonia formed by breakdown of skeletal muscle protein to the liver, where glucose is synthesized from alanine for return to muscle, the serum free alanine levels in seals may reflect the changes in rates of gluconeogenesis regulated by liver metabolism, in response to the different dietary protein intake between the two fish diets (refer to Chapter 3).

Mirroring to the above patterns of alanine and glutamine, the levels of a number of dietary essential amino acids, including leucine, isoleucine, valine, lysine, arginine and threonine, decreased when diet was switched to pollock, as shown in Fig.6.5. Studies show that fasting FAA levels in humans are maintained primarily by skeletal muscle protein breakdown and the resulting release of amino acids to the blood (Armstrong and Stave II, 1973). Our data show that the total serum free essential amino acid (EAA) pool size in Travis represents more than 40% of the total FAA pool on a herring diet and less than 30% on a pollock diet. Because the FAA pool supplies the building blocks for protein synthesis, its EAA pool size plays an important role in understanding the nutritional regime and the effects of dietary protein intake on protein retention and turnover (Carter et al. 2000). Significant differences in daily protein intake of Travis and

Pender on herring and pollock diets (refer to Fig. 3.7) indicate that correlated changes in serum FAA levels may result from changes in liver protein metabolism in response to the varying exogenous protein intake. As discussed in Chapter 3, stable isotope analysis has demonstrated a relationship between nitrogen isotopic fractionation of harbor seals and the dietary protein intake. Relatively low protein intake on the herring diet results in lesser nitrogen fractionation in harbor seal serum, which is consistent with the assumption that main isotopic fractionation occurs in transamination or deamination processes (Gaebler et al. 1966; Macko et al. 1987).

It is well known that marine mammals adapt a high fat diet throughout their lives and that lipid is the major energy source for these species, although glucose and protein also account for a small portion of energy supply (Kirby and Ortiz 1994). Significant changes in protein intake may cause changes in partitioning of amino acids into protein synthesis or gluconeogenesis, which has been suggested as the major difference between the feeding and fasting state in elephant seals (Kirby and Ortiz 1994). Studies demonstrate that both protein synthesis and urea excretion increase with high protein intake (Sick et al. 1997). Activated gluconeogenesis may occur during relatively low protein intake on the herring diet, as evidenced by the low level of serum free alanine (Murphy and Hochachka 1981). Insulin metabolism may also play a role in regulating several free EAA levels. Decreasing levels of Val, Ile, Leu and Phe have been observed in humans after stimulation of endogenous insulin release by administration of glucose (Crofford et al. 1964; Zinneman et al. 1966). Elevated levels of Val, Ile, Leu, Phe and Tyr

have also been observed in obese humans, and their levels were significantly correlated with serum immuno-reactive insulin levels (Armstrong and Stave III 1973). However, studies have found that elephant seals are relatively insulin insensitive, an adaptation to very low dietary carbohydrate intake of this species (Kirby and Ortiz 1994).

Although significant, the changes in free EAA composition in response to the different diets are not the same for all EAA. As shown in Fig. 6.5, six of nine (His is not included due to its co-elution with Gln) EAA demonstrated patterns related to diets, but no such patterns were found in Met and Phe (plus Tyr). This can be explained by the fact that the regulation of free amino acid concentrations is not precise, and non-limiting essential amino acids may accumulate at relatively higher concentrations compared with the most limiting essential amino acids (Carter et al. 2000). For example, our data show that Phe is present at such a low level in the serum FAA pool that it is probably the most rate-limiting EAA for protein synthesis. Since Phe can only be supplied from dietary proteins, once free Phe accumulates to a sufficient concentration in the serum, it is immediately used for protein synthesis. Thus there would be no correlation observed between the diet and serum free Phe pool of harbor seals. Kaushik et al (1988) observed that a greater proportion of available free arginine was used for protein synthesis when rainbow trout were fed a very low arginine diet. Other studies have also suggested that omission of even one essential amino acid might result in a state of negative nitrogen balance.

Comparison of serum FAA among wild phocids

Many physiological factors, such as protein intake, nutritional status, physical activity before blood draw, sex and age may alter blood free amino acid profiles. As discussed earlier, dietary amino acid profiles significantly influence FAA compositions in consumers. The notable differences in valine and alanine levels among phocid seal species correspond well with their dietary diversity in different environmental regimes. Field observations and stable isotope food web analysis both demonstrate that the trophic levels of three Antarctic seal species are Weddell > Ross > crabeater (Castellini et al, unpublished data). Laws (1984) observed that krill was the primary food of crabeater seals and accounted for more than 90% of their food. Therefore, crabeater seals ranked at the primary carnivore trophic level and the mean $\delta^{15}N$ values are as low as 7‰. In contrast, Weddell seals and harbor seals are top predators with pelagic fish diets. The major food of Ross seals is squid, placing them in an intermediate trophic level. Unlike captive harbor seals, whose blood samples were collected following an overnight fast, wild seal blood samples were taken without any knowledge of the time of their last meal. Since the postprandial FAA profile can vary with time, there is an additional source of variability in FAA profiles for these wild seal species. The relatively low level of serum free alanine in Weddell seals is believed to be closely related to the high rate of gluconeogenesis (Murphy and Hochachka 1981). However, the low level in serum free alanine of harbor seals, compared to that of Ross and crabeater seals, is contrary to the results by Murphy and Hochachka (1981). They found alanine levels in whole blood that

were threefold higher in harbor seals and humans than in the Weddell seals. So far there are no other comparable data available to help explain these differences.

CONCLUSION

In summary, for captive harbor seals, serum FAA profiles correlate with diet switches between the two fish diets, indicating changes in liver metabolism in response to varying dietary protein intake. Notable differences in serum FAA compositions are observed in some wild seal species, which also seem to be linked to their diverse diets. In contrast, HAA profiles of serum and RBC proteins in captive harbor seals have almost constant compositions and reflect the consistent composition of their constituent proteins independent of dietary protein source or intake. HAA profiles in serum proteins are indistinguishable among four seal species. Comparisons of HAA profiles in fish tissue protein hydrolyzates between herring and pollock reveal similar compositions, although the THAA concentrations (μmol/g) in pollock are on average 1.5 times higher than those in herring based on dry weight.

Chapter 7

Stable Isotope Ratios in Pacific Herring (*Clupea pallasi*) and Walleye Pollock (*Theragra chalcogramma*): Individual Differences and Interannual Variations

ABSTRACT

Variations of stable carbon and nitrogen isotope ratios in Pacific herring (*Clupea pallasi*) and walleye pollock (*Theragra chalcogramma*) were investigated in order to determine the trophic enrichment of harbor seals (*Phoca vitulina*) in a controlled feeding trial. Several batches of Pacific herring and walleye pollock were caught either in Prince William Sound or the Gulf of Alaska during 1997-2000. Carbon and nitrogen isotopic composition of these two fish species was examined in relation to catch location and season, lipid content, standard body length and mass. The $\delta^{13}C$ and $\delta^{15}N$ values varied with geographic locations and different years. Positive relationships between lipid content and C/N ratio were found in herring (r^2=0.8305, n=83) and pollock (r^2=0.6574, n=43). No significant relationships were found between the isotopic composition of fish tissues and their standard body length or mass (n=93 for Pacific herring, n=54 for walleye pollock). Comparison of isotope ratios between lipid-free fish tissues and whole fish tissues revealed that lipid extraction did not greatly narrow the range of $\delta^{13}C$ values, but generally yielded elevated absolute values, indicating that variability in carbon isotope ratios among individual fish specimens was largely independent of the lipid effect. In addition, our results showed that the lipid extraction procedure might also alter nitrogen isotope ratios.

Keywords: $\delta^{13}C$; $\delta^{15}N$; Pacific herring (*Clupea pallasi*); walleye pollock (*Theragra chalcogramma*); Prince William Sound; Gulf of Alaska

INTRODUCTION

Stable isotope techniques have been widely used for tracing the organic matter flow of food webs over the past three decades. This approach is based upon the observation that the carbon and nitrogen isotopic compositions of an organism are responsive to those of its diet (DeNiro and Epstein1978; DeNiro and Epstein1981a,b; Minagawa and Wada 1984; Schoeninger and DeNiro1984; Sealy et al. 1987; Owens 1987; Peterson and Fry 1987; Rau et al. 1992; Hobson and Welch 1992; reviewed by Michener and Schell 1994). In general, a fairly predictable 3 - 5 ‰ enrichment in nitrogen isotope ratios per trophic level and a relatively conservative 0 - 1 ‰ trophic enrichment in carbon isotope ratios are the basis of these applications. The key to using stable isotope analysis to accurately define the trophic relationships in an ecosystem is to have a comprehensive understanding of the factors that may affect isotopic fractionation between consumers and their prey species. Studies have demonstrated a wide range of intra-species and inter-species variability and substantial overlaps, especially for carbon isotope ratios, which complicate the trophic structure analysis and dietary reconstruction. For example, laboratory controlled experiments have shown that trophic fractionation can a) vary in different species (DeNiro and Epstein 1978; 1981; Hobson and Clark 1992a, b; Focken and Becker 1998); b) vary within one species under different physiological conditions such as fasting or starvation (Hobson and Clark 1993; Best and Schell 1996)

and c) change in the same species in response to different diets (Hobson and Clark 1992a,b; Focken and Becker 1998, Chapter 3). Therefore appropriate data interpretation requires knowledge of the trophic fractionation that occurs during food assimilation and metabolism in consumers and the variability of isotope ratios in prey species.

In this laboratory controlled feeding trial, captive harbor seals (*Phoca vitulina*) were used as an experimental model, with Pacific herring (*Clupea pallasi*) and walleye pollock (*Theragra chalcogramma*) as representative prey species, to test how metabolic and physiological processes in harbor seals affect the isotopic fractionation in response to different diets. $\delta^{13}C$ and $\delta^{15}N$ variations of Pacific herring and walleye pollock were examined in relation to potentially controlling factors such as catch location and season, fish tissue composition, and relative fish body size.

MATERIALS AND METHODS

Analysis of variance (one-way ANOVA, Tukey-Kramer pairwise comparisons) was conducted to compare isotope ratios among different batches of Pacific herring and walleye pollock. Paired t-tests were used to determine if differences existed in carbon and nitrogen isotope ratios between whole fish and lipid-free fish tissues. Sample collection and preparation methods are detailed in section Diet Fish Sampling and Analysis in Chapter 2.

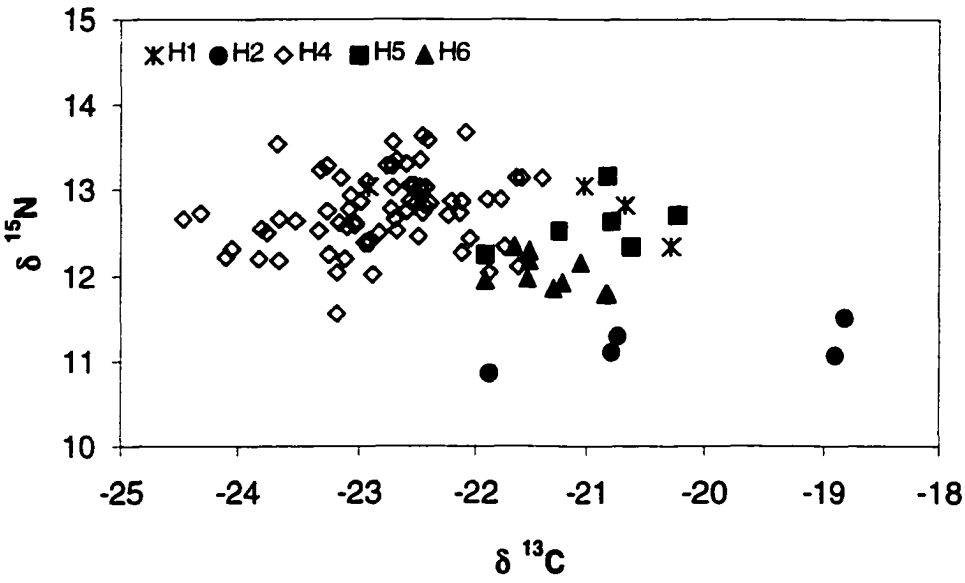
RESULTS

Carbon isotope ratios

Carbon and nitrogen isotope ratios in all batches of herring and pollock are shown in Fig. 7.1a, b. The mean ± SD of $\delta^{13}C$ and $\delta^{15}N$ values, catch locations and dates, and sample sizes are summarized in Table 7.1. In general, $\delta^{13}C$ values in herring spanned a much wider range than those in pollock, whereas $\delta^{15}N$ values were less variable in herring than in pollock. This is reasonable since the lipid content in herring is much more variable than that in pollock. Proximate analysis revealed that lipid contents ranged from 15.6% to 60.7% in herring and 10.8% to 32.9% in pollock (based on dry weight). Positive relationships between lipid content and C/N ratios were found in both herring (r^2=0.8305, n=83) and pollock (r^2=0.6574, n=43) (Fig. 7.2a, b), indicating that C/N ratios might be useful for estimating lipid contents in both fish species. Similar plots of carbon and nitrogen isotope ratios against C/N ratios (Fig. 7.3a, b) showed that a weaker negative relationship existed for $\delta^{13}C$ in herring (r^2=0.6515, n=83), while no significant relationship existed for pollock or nitrogen isotope ratios. This suggested that roughly 60% of the variability in the $\delta^{13}C$ values in herring was attributable to varying lipid contents in individual herring specimens, since lipids are generally depleted in $\delta^{13}C$ by 6‰ relative to the whole fish tissues (DeNiro and Epstein 1978; Tieszen et al. 1983).

The effects of lipid content on carbon and nitrogen isotope ratios were further examined by comparing $\delta^{13}C$ and $\delta^{15}N$ values between whole fish tissues and lipid-free fish tissues in a subset of fish specimens (Fig. 7.4a, b). The results showed that lipid-free

a)



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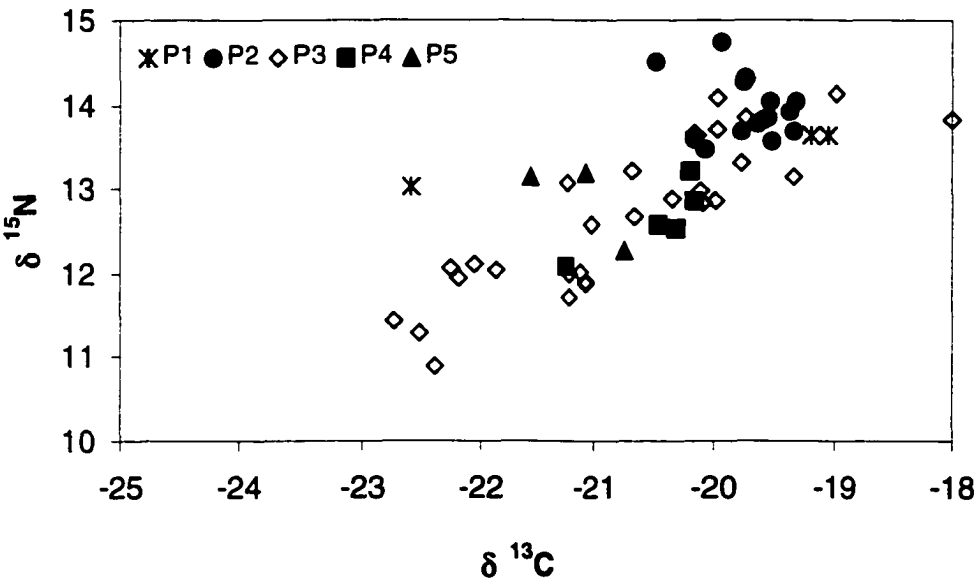


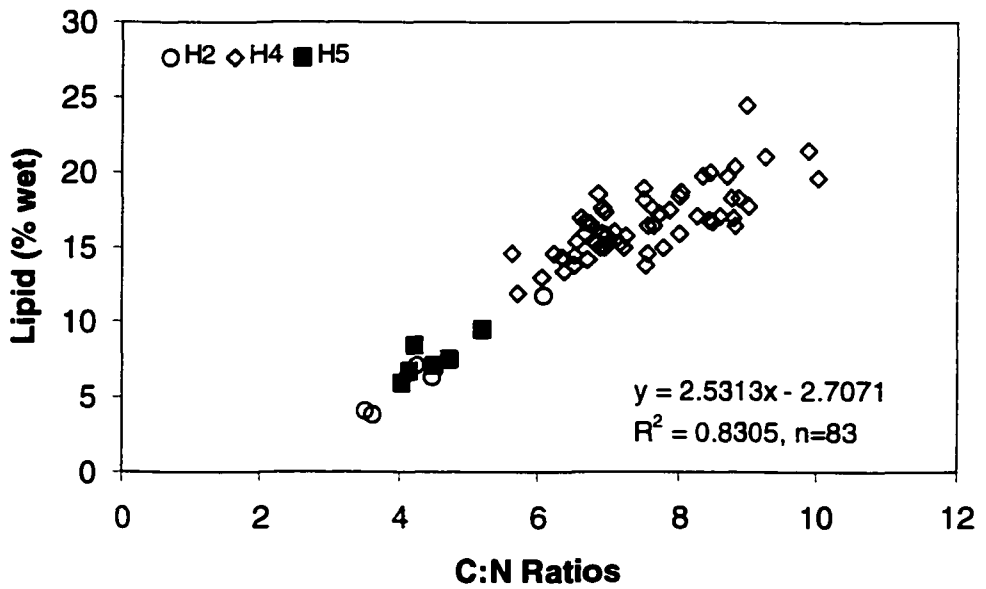
Fig. 7.1 Variations of carbon and nitrogen isotopic compositions in a) herring and b) pollock.

Table 7.1 Carbon and nitrogen isotope ratios (mean ± SD), and catch locations and time of different batches of Pacific herring and walleye pollock

Batches	Sample size N	$\delta^{13}C$ (‰) Mean ± SD	$\delta^{15}N$ (‰) Mean ± SD	Catch Location/Date
H1	6	-20.90 ± 1.03	12.65± 0.43	PWS, Nov. 97
H2	6	-20.22 ± 1.33	11.16 ± 0.24	Atlantic Ocean
H4	72	-22.82 ± 0.72	12.76 ± 0.44	PWS, Nov. 98
H5*	6	-20.94 ± 0.58	12.60 ± 0.33	Unknown
H6	10	-21.34 ± 0.36	12.02 ± 0.21	Petersburg, Dec. 99
P2	15	-19.72 ± 0.32	13.96 ± 0.37	GOA, Mar. 98
P3	30	-20.75 ± 1.14	12.68 ± 0.89	GOA, Jan 99
P4	6	-20.38 ± 0.46	12.88 ± 0.65	Cordova, Mar. 99
P5	6	-21.13 ± 0.41	12.86 ± 0.52	Unknown

* Juvenile herring for bait, standard length < 150mm.

a)



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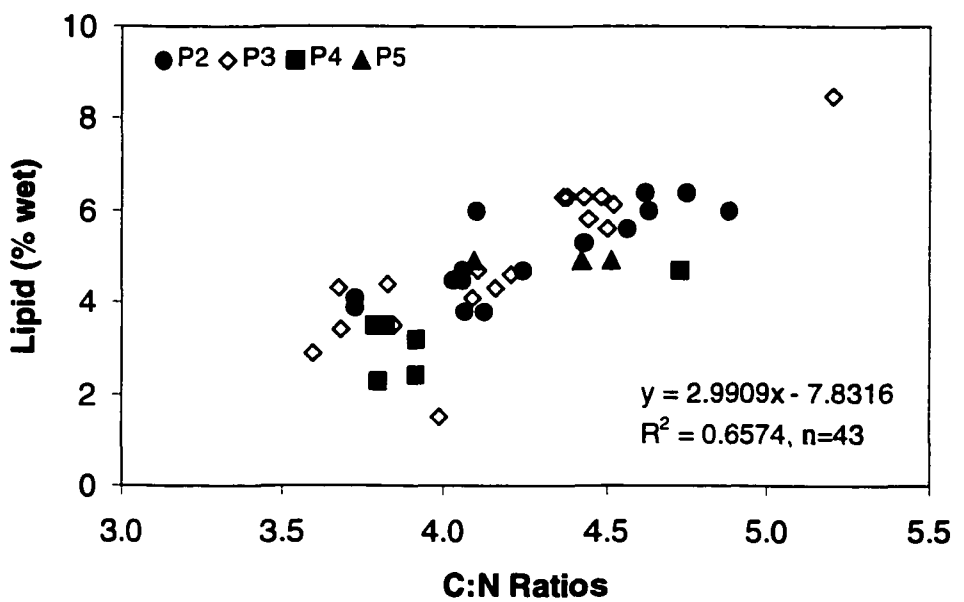
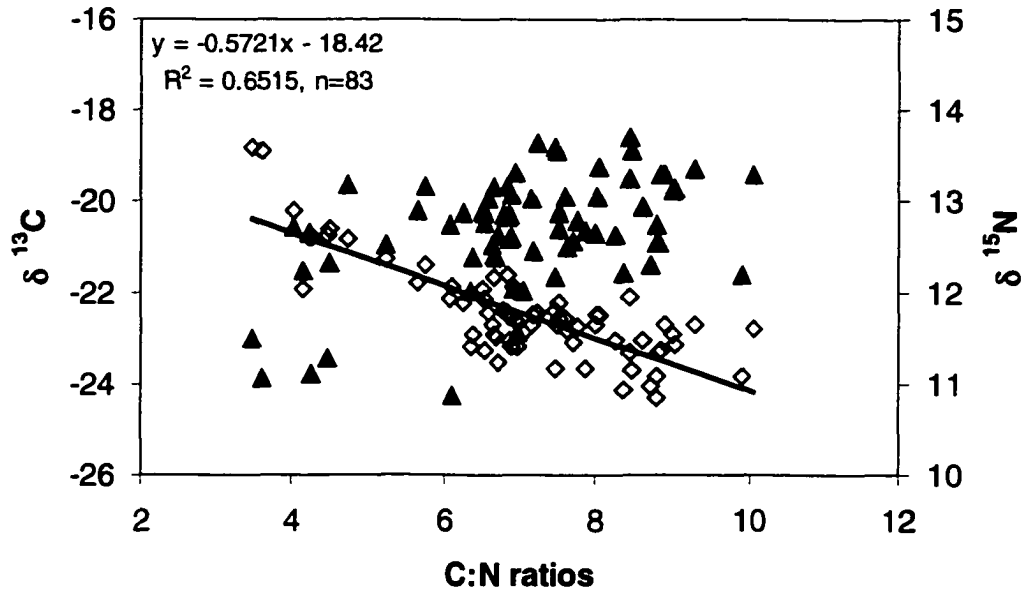


Fig. 7.2 The relationship between the lipid contents (%, wet weight) and C: N ratios in a) herring and b) pollock.

a)



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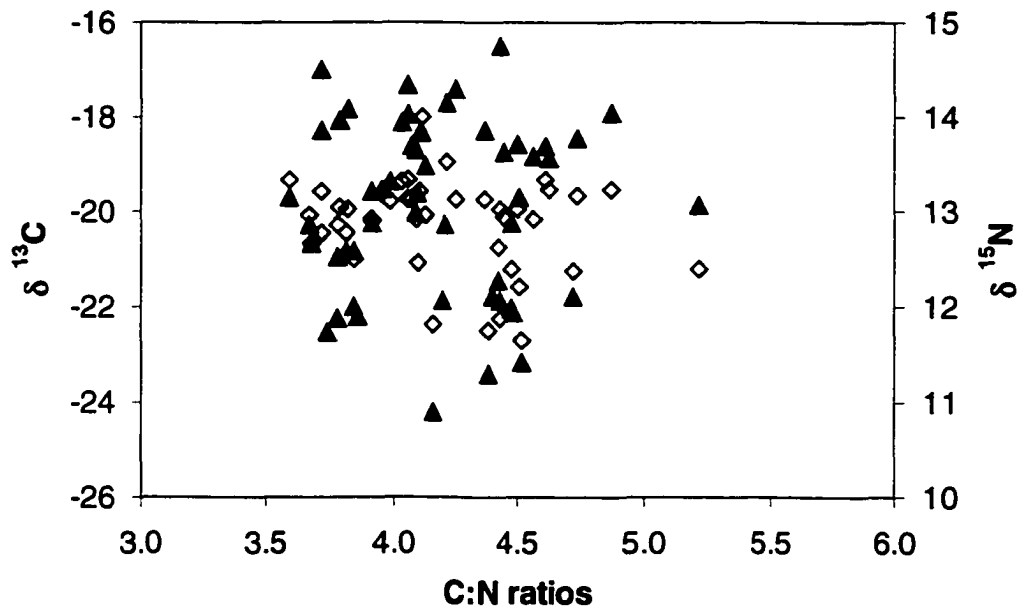


Fig. 7.3 The relationship between carbon (◇) and nitrogen (▲) isotope ratios and C: N ratios in a) herring and b) pollock.

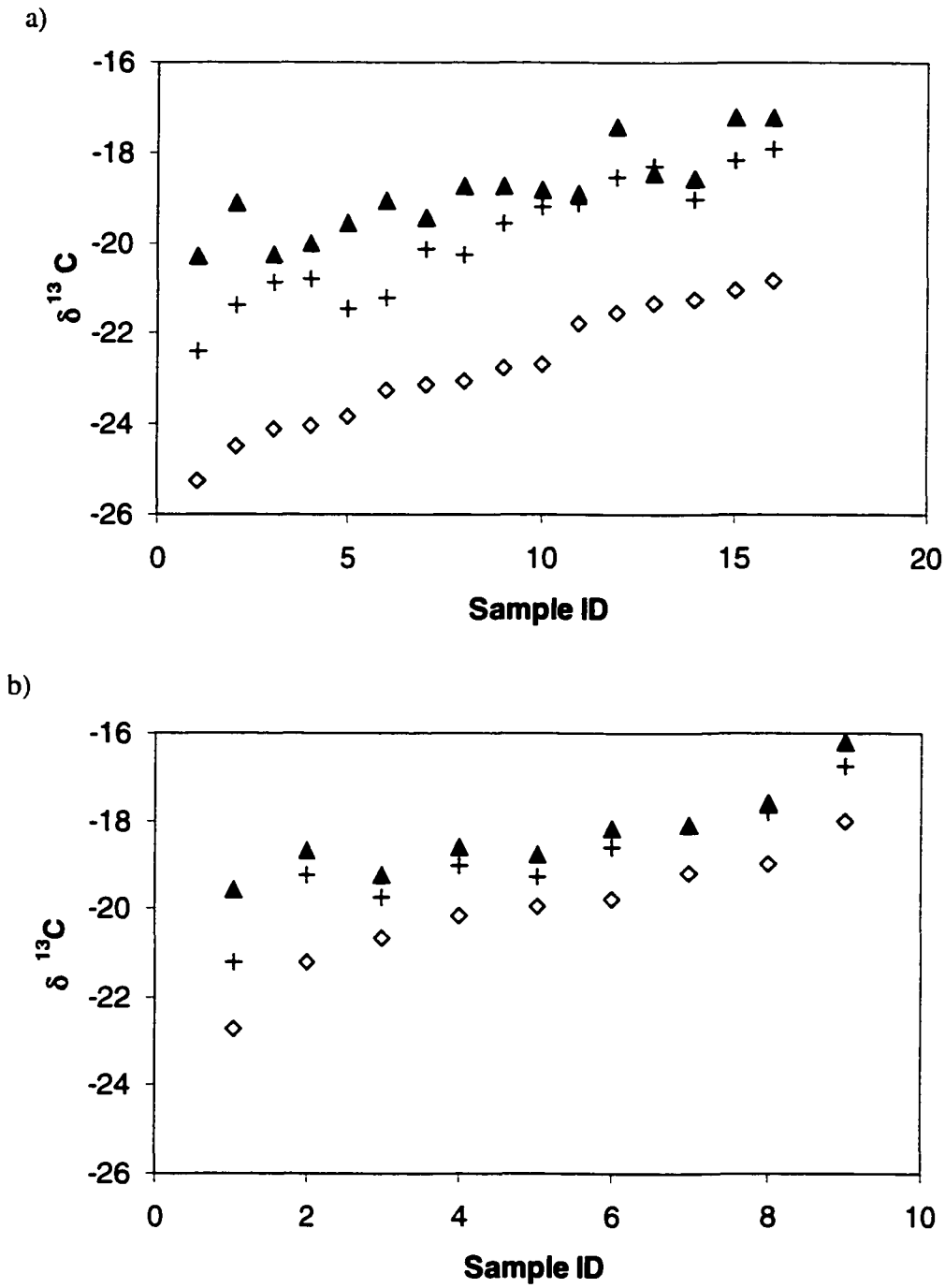


Fig. 7.4 Comparison of carbon isotope ratios between lipid-free fish tissues (▲) and whole fish tissues (◇). Normalized lipid-free $\delta^{13}C$ values (+) based upon lipid contents are also shown. a) herring and b) pollock.

$\delta^{13}C$ values in herring were more enriched on average by 3.90‰ over those of whole herring tissues (n=16, paired t-test, $p < 0.05$). Lipid-free $\delta^{13}C$ values in pollock were generally 1.74 ‰ greater than those in whole pollock tissues (n=9, paired t-test, $p < 0.05$). The range of $\delta^{13}C$ values in herring tissues decreased 1.36‰ with lipid removal; $\delta^{13}C$ values spanned 4.44‰ in whole tissues and 3.08‰ in lipid-free tissues. There was no significant change in the variability of $\delta^{13}C$ values in pollock. Differences in diet, age, habitats, metabolism or other characteristics of individual specimens might contribute to the variability of the $\delta^{13}C$ values in both fish species. The measured lipid-free $\delta^{13}C$ values were similar to the normalized $\delta^{13}C$ values, calculated by subtracting 6‰ x lipid content (% dry weight) from the whole fish tissue $\delta^{13}C$ values (Alexander et al. 1996). As shown in Fig. 7.4a, b, most data were in good agreement with normalized $\delta^{13}C$ values, and there were no statistically significant differences between the measured lipid-free $\delta^{13}C$ values and normalized values in either fish species (paired t-test, $p < 0.05$). Moreover, a positive correlation (r^2=0.8451, n= 16) was found between ($\delta^{13}C_{\text{lipid-free}} - \delta^{13}C_{\text{whole fish}}$) and (C:N ratio $_{\text{whole fish}}$ - C:N ratio $_{\text{lipid-free}}$) in herring (Fig. 7.5), which demonstrated that the larger the differences in C:N ratios between lipid-free and whole herring tissues, the greater the lipid effect on $\delta^{13}C$ values of herring. No such relationship was observed in pollock.

Nitrogen isotope ratios

As shown in Fig. 7.1b, $\delta^{15}N$ values in pollock ranged from 10.89 to 14.75 ‰, spanning more than one full trophic level even in the same batch of pollock (P3). The

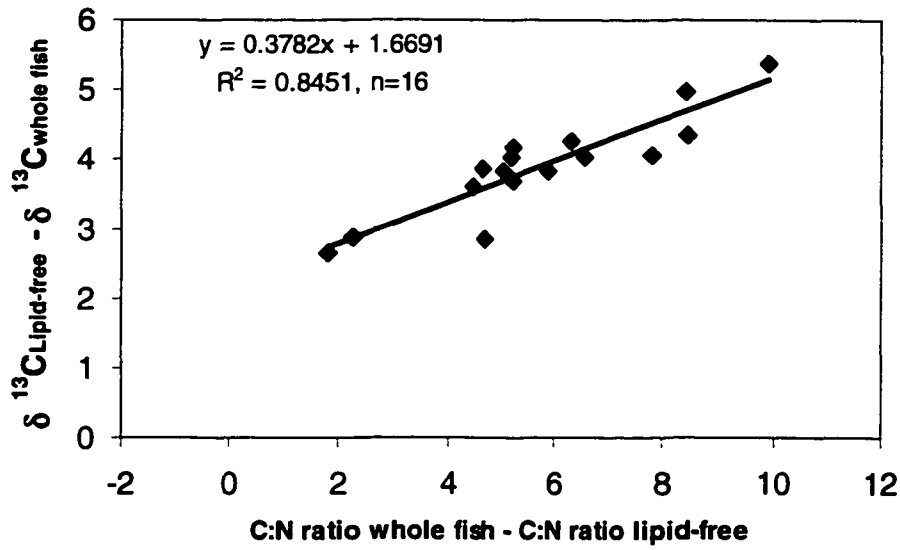


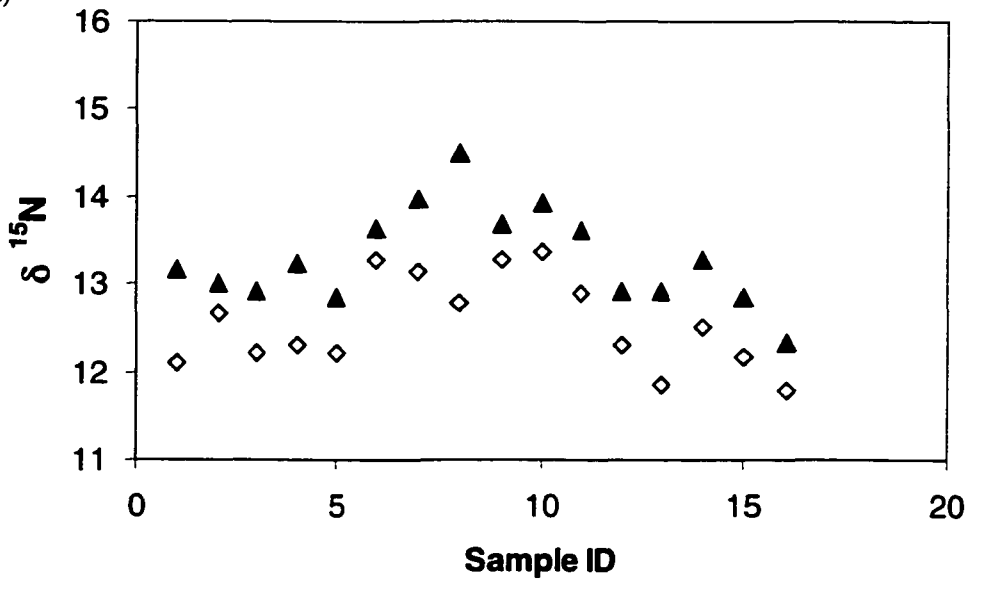
Fig. 7.5 The correlation between ($\delta^{13}C_{\text{lipid-free}}$ -$\delta^{13}C_{\text{whole fish}}$) and ($C{:}N\ \text{ratio}_{\text{whole fish}}$ - $C{:}N\ \text{ratio}_{\text{lipid-free}}$) in herring.

$\delta^{15}N$ values seemed to co-vary with the $\delta^{13}C$ values. Unlike carbon isotope ratios, nitrogen isotope ratios are usually not affected by lipid contents in fish tissues. No relationship existed between ($\delta^{15}N_{\text{lipid-free}} - \delta^{15}N_{\text{whole fish}}$) and (C: N ratio $_{\text{whole fish}}$ - C: N ratio $_{\text{lipid-free}}$). In fact, $\delta^{15}N$ values were less variable in herring than pollock, despite higher and more variable lipid contents. However, a significant difference in $\delta^{15}N$ values was found between lipid-free and whole fish tissues (paired t-test, $p < 0.05$), indicating that the lipid extraction procedure altered the nitrogen isotope ratios. Our data showed that lipid extraction increased the $\delta^{15}N$ value an average of 0.77‰ (range 0.29‰ - 1.71‰) in both pollock and herring (Fig. 7.6a, b). Lipid extraction did not decrease variability of $\delta^{15}N$ values in either pollock or herring. Fig. 7.7a, b, c, d show the relationships between $\delta^{15}N$ values of fish tissues and body standard length or mass. Neither of these variables appeared to affect the nitrogen isotope ratios in either herring or pollock.

Regional and year to year isotope variations

Statistically significant intraspecies and interspecies differences of $\delta^{13}C$ and $\delta^{15}N$ were found with catch location and time (Fig. 7.8a,b,c). Analysis of variance (one-way ANOVA, Tukey-Kramer's pairwise comparisons) was conducted to compare isotope ratios among different batches caught at different locations and times of a single species (herring or pollock) and among batches of two fish species (herring vs. pollock) used in the controlled feeding trial on the captive harbor seals. The mean $\delta^{13}C$ value of H4 significantly differed from all other herring batches (H1, H2, H5 and H6) (ANOVA,

a)



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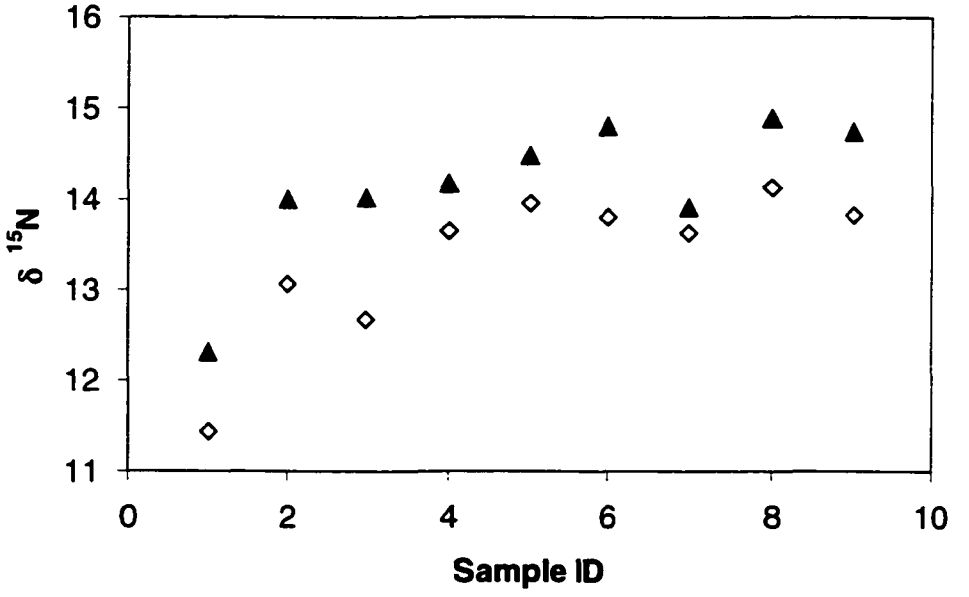
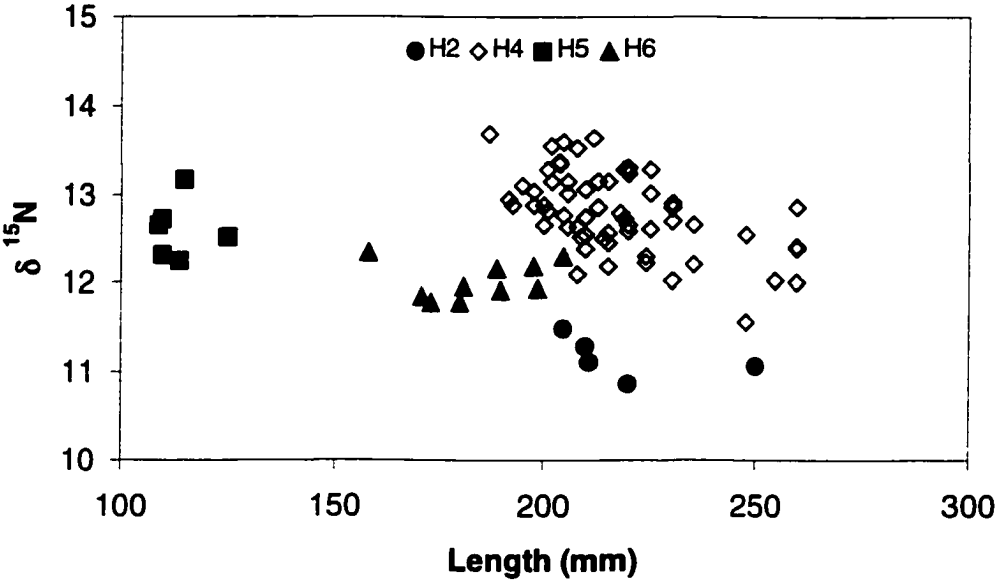


Fig. 7.6 Comparison of nitrogen isotope ratios between lipid-free fish tissues (▲) and whole fish tissues (◇). a) herring and b) pollock.

a)



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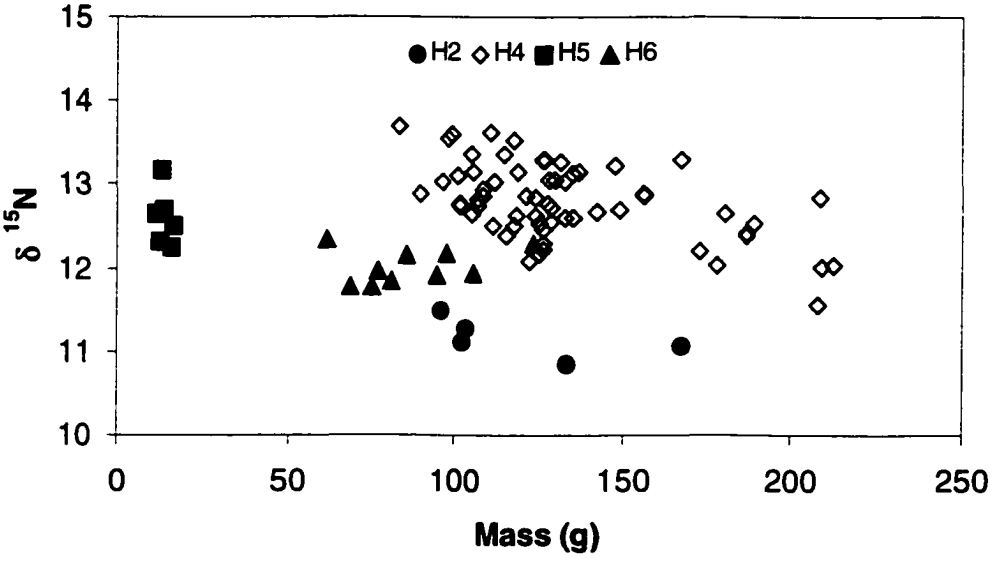


Fig. 7.7a, b Variations of nitrogen isotope ratios in herring with a) standard body length and b) body mass.

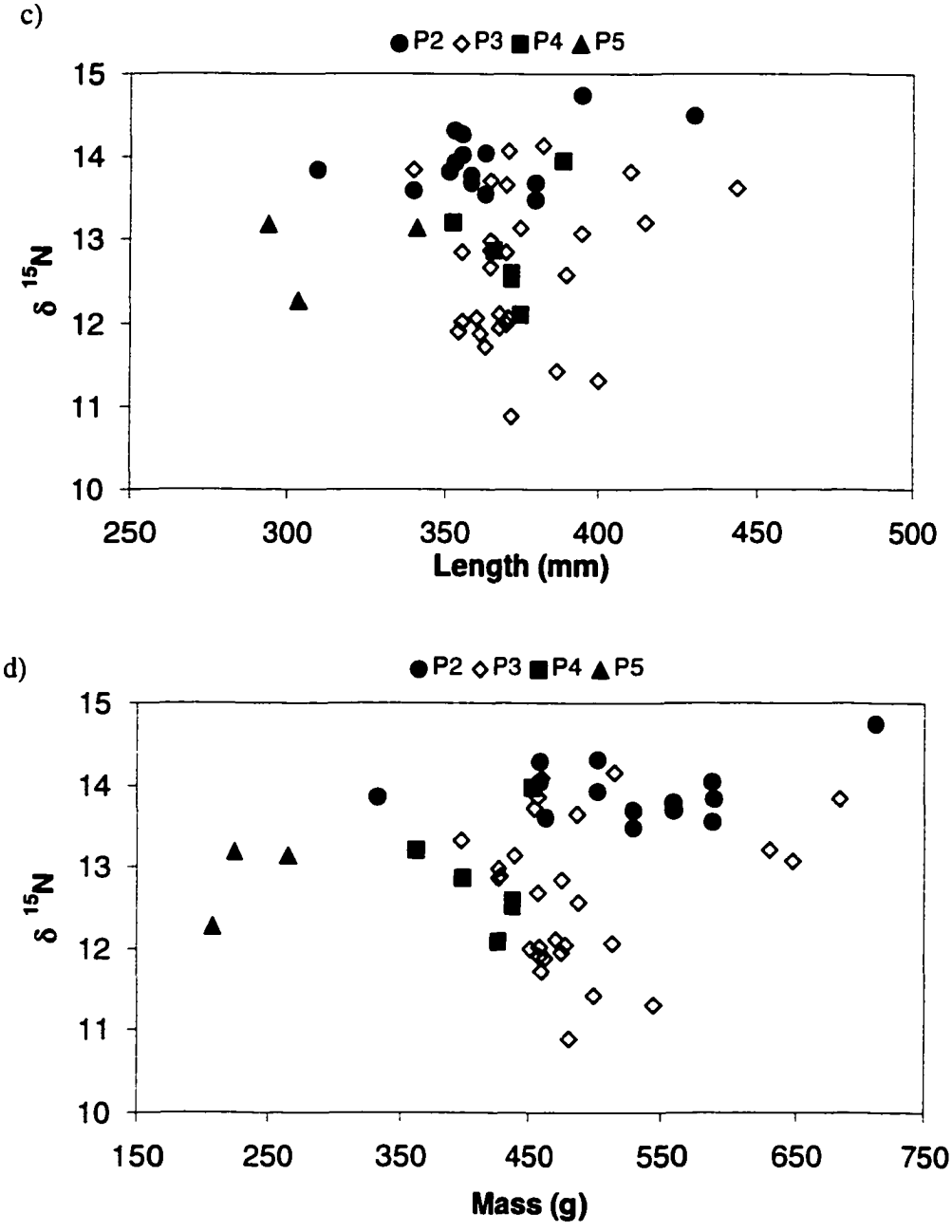


Fig. 7.7 Variations of nitrogen isotope ratios in pollock with c) standard body length and d) body mass.

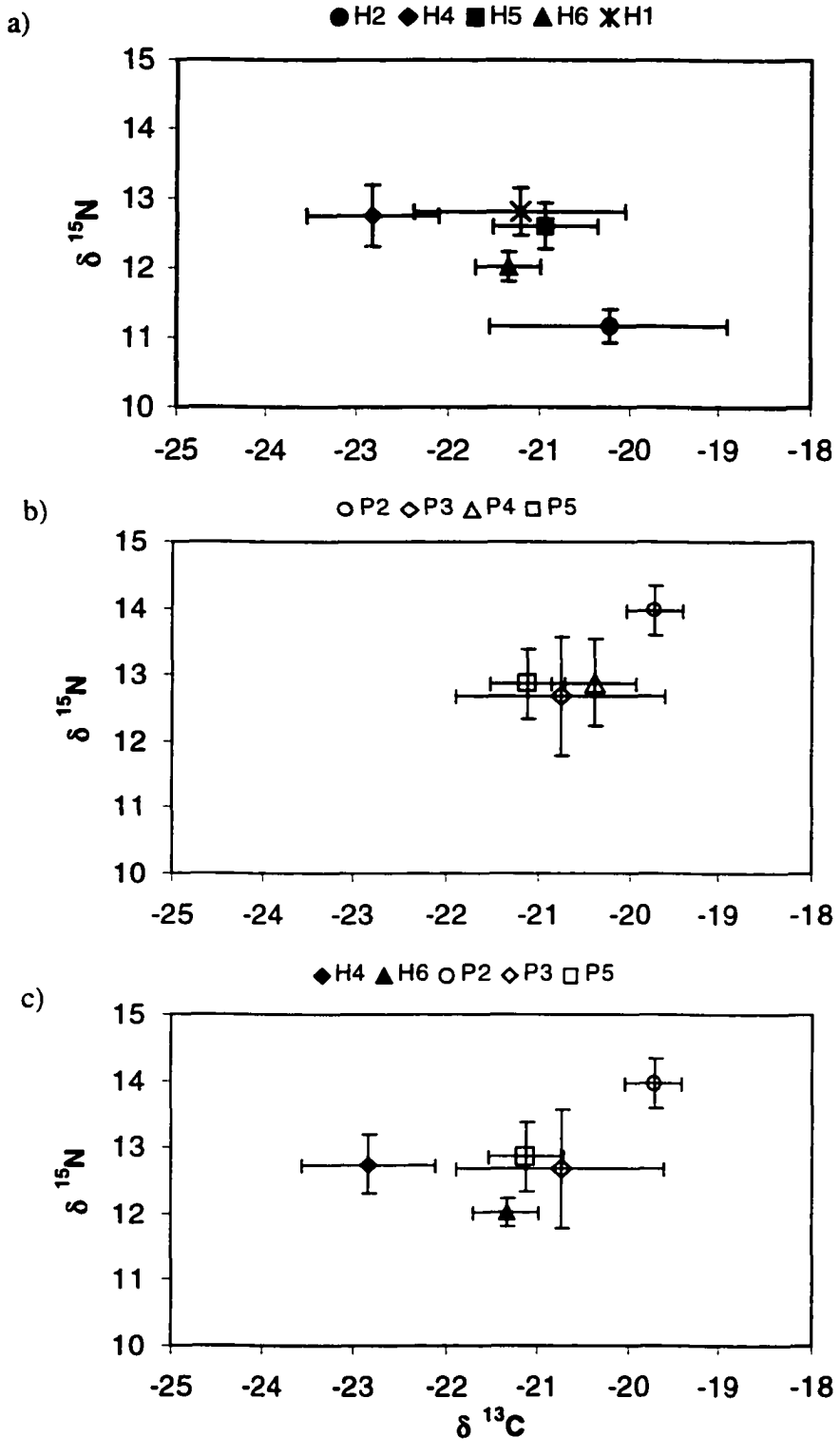


Fig. 7.8 Distribution of carbon and nitrogen isotope ratios (mean ± SD) of different batches of fish species. a) herring; b) pollock and c) paired diets used in the controlled feeding trial .

$F_{4,94} = 32.90$, $p < 0.0001$,Tukey-Kramer). There were no significant differences in mean $\delta^{15}N$ values among H1, H4 and H5 (ANOVA, $F_{4,94} = 22.34$, $p < 0.0001$, Tukey-Kramer), even though all H5 specimens were juvenile, with roughly 10 times smaller body size than H1 and H4. However, the mean $\delta^{15}N$ value of H4 differed significantly from that of H6. The significant differences in both $\delta^{13}C$ and $\delta^{15}N$ between herring H4 and H6 reflected regional or interannual differences, since H4 was caught in November, 1998 in PWS and H6 in December 1999 from near Petersburg, southeastern Alaska. Proximate analysis showed that lipid contents were similar in H4 and H6, but higher than those in H2 and H5 (Fig. 7.8a). For pollock, only the mean $\delta^{15}N$ value of P2 was significantly different from those of other pollock batches (P1, P3, P4 and P5) (ANOVA, $F_{4,51} = 7.93$, $p < 0.0001$, Tukey-Kramer). The mean $\delta^{13}C$ value of P2 differed from that of P3, but did not significantly differ from that of other batches of pollock (ANOVA, $F_{4,51} = 3.26$, $p = 0.0186$, Tukey-Kramer). Since P2 and P3 were both caught from GOA in March 1998 and January 1999, respectively, and the lipid contents were similar, the significant differences in isotope ratios between P2 and P3 were most likely caused by changes in phytoplankton or prey items between the two years.

Statistical analysis of data from five batches of two fish species (H4, H6, P2, P3 and P5) produced three distinct groups for both carbon and nitrogen isotope ratios. The highest $\delta^{13}C$ and $\delta^{15}N$ values occurred in P2, with intermediate values in P3, P5 and H6 in carbon ratios and P3, P5 and H4 in nitrogen ratios. H4 and H6 had the lowest $\delta^{13}C$ and

$\delta^{15}N$ values, respectively (for carbon ratios: ANOVA, $F_{4, 124} = 71.34$, $p < 0.0001$, Tukey-Kramer; for nitrogen ratios: ANOVA, $F_{4, 124} = 21.04$, $p < 0.0001$, Tukey-Kramer).

DISCUSSION

Effects of lipid contents

Lipid contents affect $\delta^{13}C$ values in both herring and pollock. Although a relationship exists between $\delta^{13}C$ values of whole fish tissues and C:N ratios in the fatty fish herring (r^2=0.6515, n=83), lipids alone cannot explain all the intra- or inter-species variability in $\delta^{13}C$ values. Intra-species variability is not reduced much by lipid extraction, as evidenced by comparing carbon isotopic composition between lipid-free fish tissues and whole fish tissues. The lipid extraction procedure appears to have altered the nitrogen isotopic composition in both herring and pollock tissues. This might be caused by the solvent chosen for lipid extraction. Radin (1981) noted that chloroform/methanol extraction could remove significant amounts of protein from some fish species.

Effects of body length or mass

Some studies have found positive relationships between $\delta^{15}N$ values and relative body size in several fish species (Beaudoin et al. 1999; Kline et al. 1998; Gu et al. 1996). Our data show no strong relationship between $\delta^{15}N$ values and either body length or mass. This is probably because all the pollock specimens in this study were all adult. Small differences in standard length or body mass among adult fish are not good

indicators of age. For example, specimens in P2, P3 and P4 were in the age 4+ group and specimens in P5 in the age 3-4 group, according to age classification methods based on standard length and mass of walleye pollock proposed by Incze et al. (1988). Standard length or body mass cannot distinguish above age 4 among adult walleye pollock. Standard length or body mass in most batches of Pacific herring specimens in this study (H1, H2, H4 and H6) were relatively uniform except for the much smaller H5. Overman and Parrish (2001) demonstrated that the effects of metabolic processes associated with age had a greater influence than body length or mass on $\delta^{15}N$ values of long-lived lake Walleye. Up to 3‰ differences in $\delta^{15}N$ composition (refer to Fig. 7.1b) in the same batch of pollock in this study may be related to changes in diet or metabolic fractionation associated with age.

Effects of different catch locations and years

The $\delta^{13}C$ and $\delta^{15}N$ values of this study were within the ranges of the literature data measured in juvenile Pacific herring and walleye pollock from PWS in 1994 and 1995 (Kline 1999). Significant differences in $\delta^{13}C$ and $\delta^{15}N$ values between herring batch 4 (H4) and batch 6 (H6) reflect the regional or year to year variations. Intraspecies variations in isotopic composition may occur due to a) changes in isotopic composition from the food web base (primary producers), which can be passed on to higher trophic levels in an ecosystem; b) switches in prey species or changes in foraging behaviors, which can also alter isotopic composition of consumers by changing the relative contribution of near or offshore prey species (reviewed by Michener and Schell 1994);

and c) metabolic or physiological isotopic fractionation effects on individual specimens, e.g., age, proximate composition variations due to seasonal fasting, etc. Proximate analysis showed the lipid contents of these two batches were similar, although they were caught in different areas and years (Nov. 1998, PWS, AK for H4 and Dec. 2000, Petersburg, AK for H6). However, distinct geographic gradients in zooplankton isotopic composition, which were first identified in the Beaufort and Chukchi seas (Saupe et al. 1989) and later documented in the Bering Sea and Aleutian Islands (Schell et al. 1998) as well as within PWS and the adjacent GOA (Kline 1999), may explain such intraspecies isotopic differences. Herring feed primarily on zooplankton, such as copepods, euphausids and decapod larvae, to build fat reserves (Gross and Hey 1988). Significant differences in carbon and nitrogen isotope ratios were also found between P2 and P3, despite similar lipid contents, locations and seasons of capture. These differences are most likely caused by changes in isotopic composition of primary producers between these two different years. Childers et al. has documented a significant shift in nutrient conditions in the Gulf of Alaska between 1998 and 1999. Studies have demonstrated that interannual changes in nutrient conditions, coupled with changes in water column structure in PWS and GOA, have greatly affected the growth and the timing of phytoplankton blooms as well as the rest of food webs. Seasonal variations of carbon isotope ratios observed in juvenile herring and pollock from PWS revealed that interannual changes in carbon isotope ratios of zooplankton occurred frequently in PWS (Kline 1999).

CONCLUSION

The $\delta^{13}C$ and $\delta^{15}N$ values in Pacific herring and walleye pollock varied with geographic locations and different years due to the varying isotopic composition of the base food webs. No significant relationships were found between the isotopic composition of fish tissues and their standard body length or mass. Lipid extraction did not greatly decrease the range of $\delta^{13}C$ values, but generally yielded elevated absolute values, indicating that in carbon isotope ratios among individual fish specimens was largely independent of the lipid effect. Results also showed that the lipid extraction procedure employed altered nitrogen isotope ratios.

Chapter 8

Stable Isotope Ratios in Harbor Seal *(Phoca vitulina)* Vibrissae: Growth Rates and Indicators of Diet

ABSTRACT

Growth rates and patterns of captive harbor seal (*Phoca vitulina*) vibrissae (whiskers) were studied using ^{15}N-labeled amino acid tracers. The use of vibrissae as long-term diet indicators were evaluated during a two-year controlled feeding trial conducted at the Alaska SeaLife Center in Seward, Alaska. Captive harbor seals were infused ^{15}N-labeled amino acid tracers to mark the keratinous tissues in vibrissae and allow tracing their growth rates and patterns. Results indicated that harbor seals rapidly replaced their vibrissae during summer (May to June). New vibrissae started growing in late May, and the growth rates of new vibrissae were estimated at up to 0.78 mm/day. Most new-growth vibrissae were longer than 6 cm by September, and then grew at a much slower rate during winter and spring. An average growth rate of 0.075 mm/day was obtained from one vibrissa during a period from December to the following May. $\delta^{13}C$ and $\delta^{15}N$ values in vibrissae co-varied, and reflected the diets of harbor seals, particularly during the rapid growth period. Vibrissae, which can be sampled via minimally invasive protocols, might be useful to track late spring to summer feeding patterns of harbor seals.

Keywords: Harbor seals; vibrissae; growth rates; $\delta^{13}C$; $\delta^{15}N$; diet indicators

INTRODUCTION

Harbor seals (*Phoca vitulina*) are small, non-migratory pinnipeds typically found in coastal waters over continental shelves. Harbor seals are widely distributed throughout the Northern Hemisphere but their population in western Alaska has declined significantly over the past three decades (Pitcher 1990; Frost et al.1994). Recent findings have revealed that well developed, movement-sensitive vibrissae (whiskers) of harbor seals allow them to navigate and forage for food in murky and dark waters (Dehnhardt and Kaminski 1995; Dehnhardt et al. 1998). There is rich supply of sensory nerves at the base of each vibrissa. Due to their keratinous structure and biological inertness, stable isotope ratios in vibrissae have been used as a proxy to track long-term records of diets or habitat usage of Steller sea lions and harbor seals (Hirons et al. 2001). They found that the major isotopic variations along vibrissae of Steller sea lions reflected annual oscillations due to changes in foraging locations or prey species, since the vibrissae of Steller sea lions grow constantly and are retained from year to year. In contrast, harbor seal vibrissae grow irregularly and are possibly shed annually, and they concluded that harbor seal vibrissae grew from fall to spring but ceased growing in June, with an average growth rates of 0.33mm/day (Hirons et al. 2001). Data were limited, however, and for better interpretation of natural isotopic variations along the vibrissae of wild harbor seals, an additional study of their growth patterns was undertaken.

As part of an ongoing study of harbor seal nutrition and health, including a controlled feeding trial and concurrent ^{15}N-labeled amino acid tracer experiments, we

collected captive harbor seal vibrissae to a) test the hypothesis that natural isotope variations along the length of metabolically inactive vibrissae can be used to indicate the isotopic composition of diet over periods up to a year, since the diets of the captive harbor seals were known and well controlled; and b) trace vibrissa growth rates and patterns by measuring the position of ^{15}N marked keratinous tissues in vibrissae following amino acid tracer infusions. This Chapter presents the results of analyses of 14 vibrissae from four captive harbor seals.

MATERIALS AND METHODS

Vibrissae are composed of a high percentage of glycine residues that are essential to the tight, α-helical keratinous structures. Hence, glycine was used as the preferred amino acid tracer, although ^{15}N-labeled valine, leucine and phenylalanine were also used in the second tracer experiment. Refer to Controlled Feeding Trial, ^{15}N-labeled Amino Acid Tracer Experiments and Harbor Seal Vibrissa Sampling and Stable Isotope Analysis in Chapter 2 for the details of method.

RESULTS

Vibrissa growth patterns

Fig. 8.1 shows the nitrogen isotope ratios of four whiskers from harbor seal Pender, sampled on 19 Dec. 1998 (day 4 after ^{15}N-labeled glycine infusion), 25 Feb. 1999 (day 72), 20 May 1999 (day 156) and 16 Sept. 1999 (day 275). The ^{15}N-labeled glycine marker was observed in the first two whiskers, but no marker was evident in whiskers

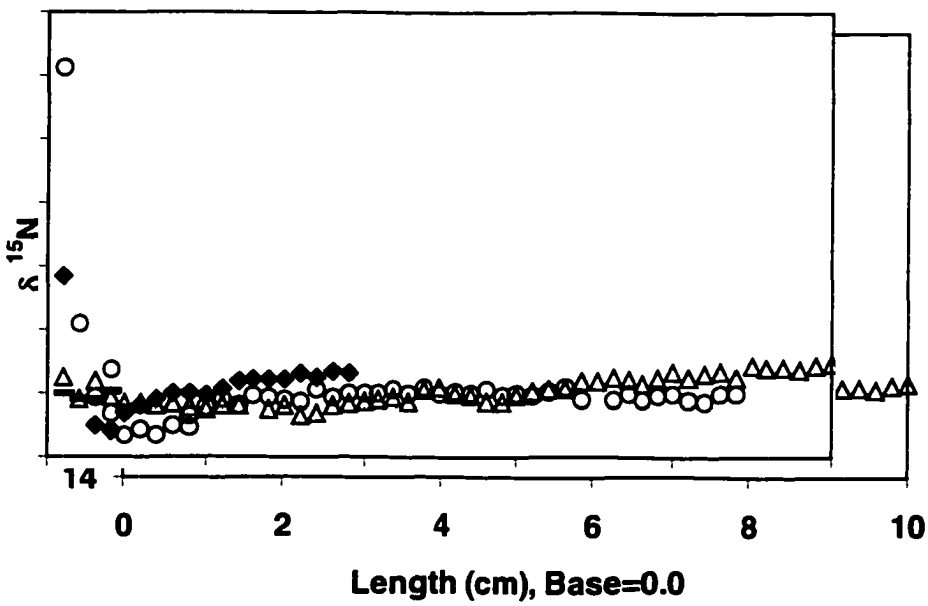
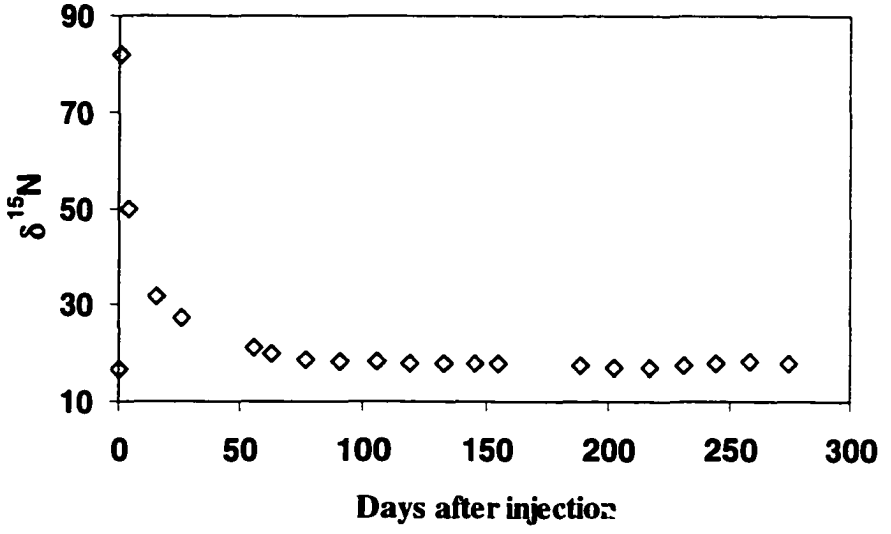


Fig. 8.1 Nitrogen isotope ratios along four whiskers from harbor seal Pender following the ^{15}N-labeled glycine infusion. Whiskers were sampled on 19 Dec. 1998 (○ day 4 after glycine infusion); 25 Feb. 1999 (◆ day 72); 20 May 1999 (▬ day 156) and 16 Sept. 1999 (△ day 275).

sampled at 156 days and 275 days after infusion. Fig. 8.2 shows the nitrogen isotope variations over time in serum and red blood cells of Pender, after the ^{15}N-labeled glycine was infused on 16 Dec. 1998. A maximum $\delta^{15}N$ value of 26.2‰ was measured near the base of the Pender whisker sampled on day 4, which was lower than that simultaneously found in serum (49.9‰) but much higher than that in red blood cells (18.0‰) sampled on the same day. An enriched $\delta^{15}N$ value of 19.7‰ was observed at the base of the second Pender whisker sampled on day 72, which was similar to the $\delta^{15}N$ value in serum (18.5‰) and red blood cells (19.4‰) sampled at the same time. The third Pender whisker was very thin and short (less than 2 cm) and assumed to be a newly grown whisker. The $\delta^{15}N$ values along the length of this whisker were all within the range of natural abundance. The fourth Pender whisker sampled on day 275 was 10 cm in length and also showed no ^{15}N enriched marker from the glycine infusion. Hirons et al (2001) suggested that harbor seals replace part of their whiskers annually and speculated that whiskers grow from fall to the following spring, but cease growing in June. Our data indicated that the whiskers sampled in late May and September might be new growth ones, based upon the finding that there was no ^{15}N enriched glycine marker evident along the length of these whiskers. As Fig. 8.2 shown, Pender serum and red blood cell pools were almost completely devoid of the ^{15}N label and had almost returned to pre-infusion baseline $\delta^{15}N$ values within approximately 100 days and 175 days following the tracer infusion. By this time, there were no ^{15}N enriched metabolic pools available for marking new growth whiskers, such as the third whisker, which is assumed to have begun growth at some time in May.

a)



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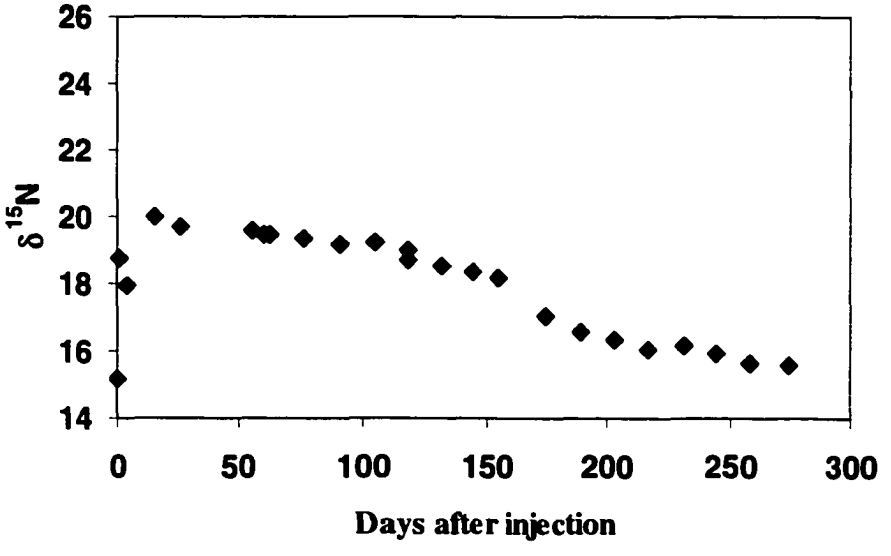


Fig. 8.2 Nitrogen isotopic compositions in a) serum (◇) and b) RBC proteins (◆) of harbor seal Pender following the ^{15}N-labeled glycine infusion.

However, in contrast to the third Pender whisker sampled on 20 May 1999 (day 156 following infusion), the whisker from Poco sampled on 18 May 1999 (day 162) showed a peak of ^{15}N enriched keratin (Fig. 8.3a). The peak $\delta^{15}N$ value reached 61.3‰ approximately 1.0 cm from the base, and gradually declined to 52.6‰ at 0.8 cm, 17.6‰ at 0.6 cm and 16.4‰ at 0.2 cm. The $\delta^{15}N$ value at the base was 15.3‰. A $\delta^{15}N$ value of 27.4‰ at 1.2 cm from the base marked the initial incorporation of ^{15}N enriched glycine into this whisker, and the $\delta^{15}N$ was similar to the value of 26.2 ‰ at the base of the Pender whisker sampled on day 4. Assuming that the glycine marker was incorporated into the whisker immediately following infusion, when the serum glycine pool was most enriched ($\delta^{15}N$ value up to 81.7‰), and that a period of 162 days had elapsed for growth of 1.2 cm of whisker, then a growth rate of 0.075 mm/day was estimated for the period from December to May. Based on this growth rate, the glycine half-life was calculated to be 47.1 days. This estimated turnover time was comparable to that found for red blood cells (see Chapter 5 for details). The peak of ^{15}N enrichment in the Poco whisker sampled in late May indicated that this 6 cm long whisker was retained for the entire period from December to the subsequent May. However, the Poco whisker sampled on 15 Sept. 1999 (day 282) showed no marker, and was assumed to have begun growth at least 100 days after the glycine infusion.

Fig. 8.4a, b shows the variations of $\delta^{15}N$ values in whiskers sampled from Pender, Snapper and Poco on 5 Sept. 2000, 77 days after a second dosage of ^{15}N-labeled essential amino acid tracers. The Pender whisker, after a ^{15}N-labeled phenylalanine infusion, and

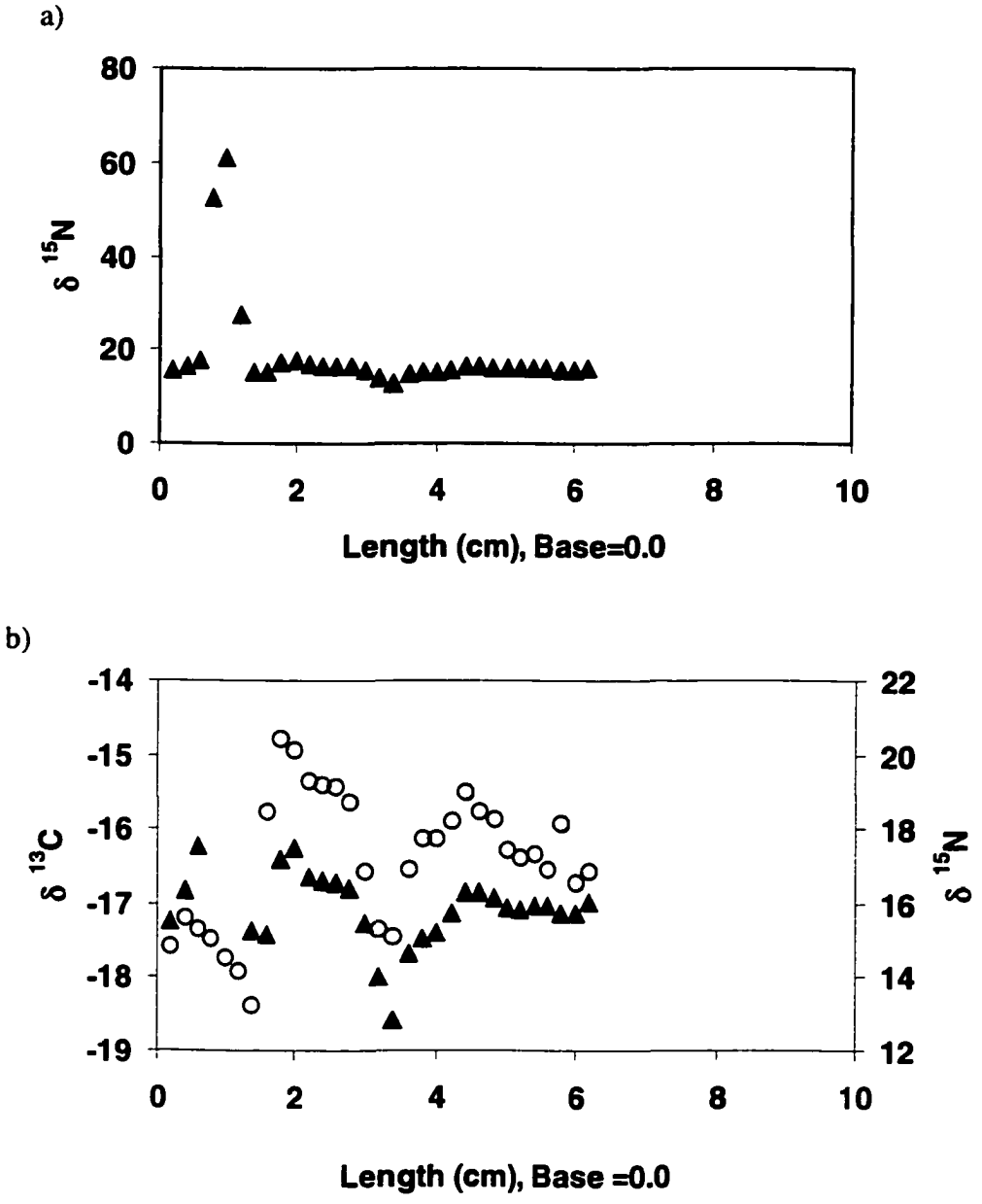
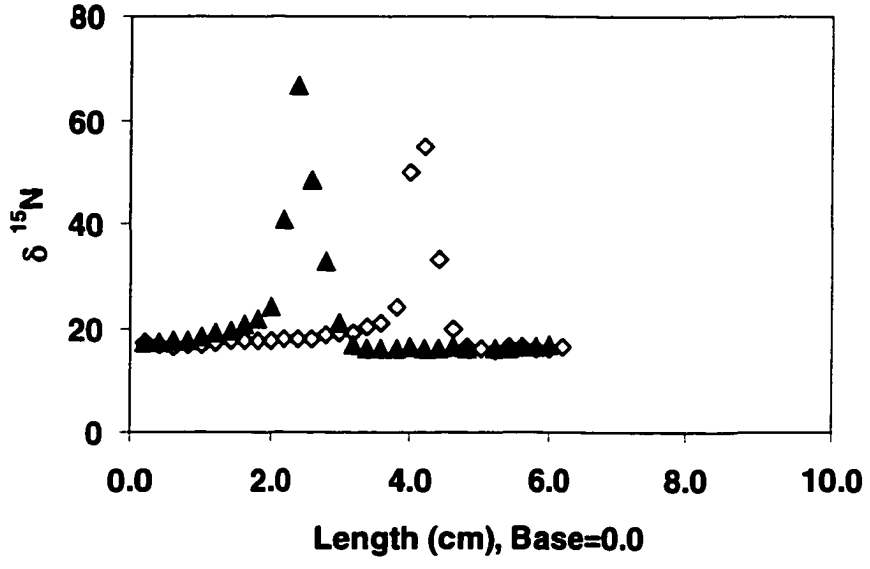


Fig. 8.3 Carbon (○) and nitrogen (▲) isotope ratios in a whisker from Poco clipped on 18 May 1999, 162 days following the ^{15}N-labeled glycine infusion. a) enrichments due to ^{15}N-labeled glycine marker; b) co-variations of natural $\delta^{13}C$ with $\delta^{15}N$ values. Note the different scales for $\delta^{15}N$ in a) and b), and that the highly enriched $\delta^{15}N$ values immediately following the glycine infusion (0.8 to 1.2 cm) are omitted from b).

a)



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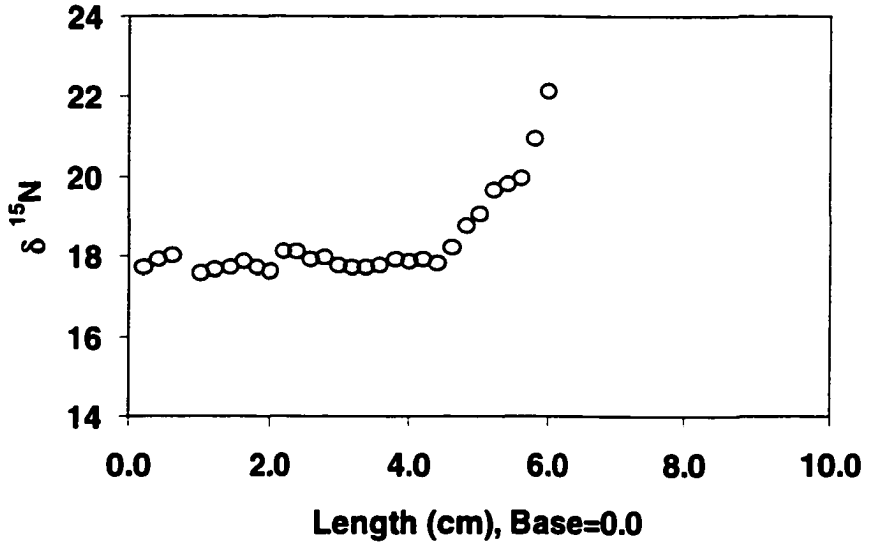


Fig. 8.4 Nitrogen isotope ratios in vibrissae from a) Pender (◇) and Snapper(▲) and b) Poco (○) sampled on 5 Sept. 2000 following the ^{15}N-labeled phenylalanine, leucine and valine infusions, respectively.

the Snapper whisker, after a ^{15}N-labeled leucine infusion, showed patterns similar to the Poco whisker sampled on 18 May 1999, after a glycine infusion. The whisker growth rates were calculated to be 0.58 mm/day for Pender and 0.36 mm/day for Snapper. The estimated tracer amino acid half-lives were 20 days for Pender and 18 days for Snapper, which were a little slower than those found in serum. Compared to the $\delta^{15}N$ patterns and peak values as high as 66.7 ‰ and 54.9 ‰ found in Snapper and Pender whiskers, the Poco whisker did not show a significant $\delta^{15}N$ peaks after a ^{15}N-labeled valine infusion, and the highest $\delta^{15}N$ value was only 22.1 ‰ at the tip of the whisker. We inferred that this was also a new growth whisker, which might have commenced growth several days after ^{15}N-labeled valine had been infused. At the time when this whisker grew, the ^{15}N enrichment of blood had been greatly reduced. Hence, the ^{15}N marker was still evident at the tip, but not in the reminder of the whisker. The estimated growth rate of this whisker was at least 0.78 mm/day.

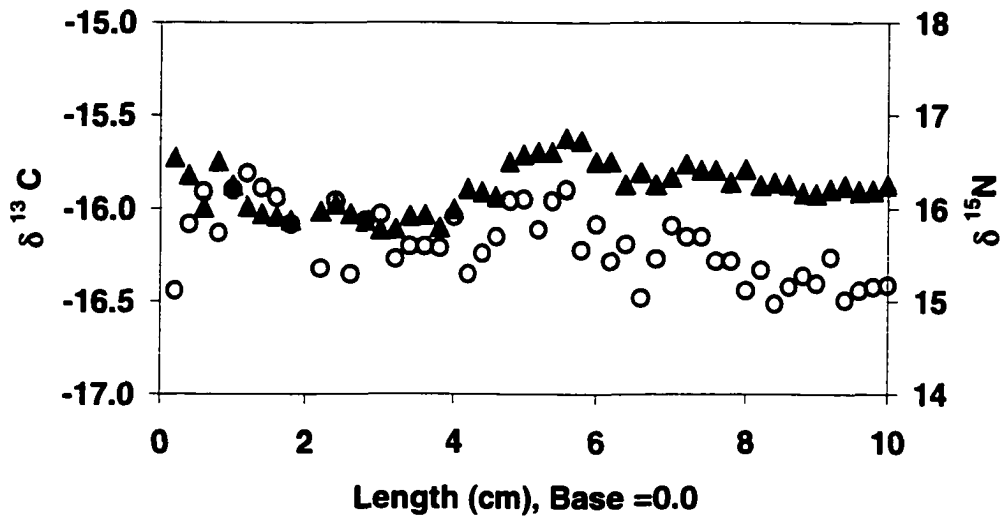
These results indicate that harbor seals lose and rapidly re-grow their whiskers during the summer season. This process might start as early as May, since no December marker was retained in the Pender whisker sampled on 20 May 1999, and as late as June, since new whiskers still grew after 21 June in Poco. The data also show that Pender replaced his whiskers earlier than Poco, since the Poco whisker sampled on 18 May 1999 still retained the previous December's marker, and some Poco whiskers had not started growth on 21 June 2000. This conclusion agrees with the field observation that harbor

seal pups in Prince William Sound shed their whiskers during their molting season in late May (Tamara Mau, personal. comm.).

Variations of vibrissa $\delta^{13}C$ and $\delta^{15}N$ values in response to diets

Variations of $\delta^{13}C$ and $\delta^{15}N$ natural abundance in harbor seal vibrissae in response to different diets were investigated. The $\delta^{13}C$ and $\delta^{15}N$ variations of two whiskers, both sampled on 15 Sept. 1999 from Snapper and Pender, are shown in Fig. 8.5a, b. No ^{15}N enriched marker from the previous December infusions was evident in either whisker. The $\delta^{13}C$ and $\delta^{15}N$ values did not vary much along the length of both whiskers, implying a constant diet isotopic composition during the period of whisker growth. This result supports, indirectly, our assumption that these whiskers are from new growth that started in late May or June. During the May-Sept. period the seals had a constant diet of either pollock for Pender or a 1:1 mixed herring and pollock diet for Snapper (refer to Table 2.1). In addition, $\delta^{13}C$ values co-varied with $\delta^{15}N$ values except for pronounced opposite variations near the bases of vibrissae, where $\delta^{13}C$ values were more depleted and $\delta^{15}N$ values more enriched than the mean values. We have no explanation for why the carbon and nitrogen isotope ratios in vibrissa bases varied in opposite directions, although a similar pattern was documented in captive harp seals (*Phoca groenlandica*, Hobson et al. 1996). The co-variations of $\delta^{13}C$ and $\delta^{15}N$ values were also observed in two whiskers sampled on 2 Dec. 1998 (Fig. 8.6a, b). Particularly in Fig. 8.6a, the variations of $\delta^{13}C$ values along this whisker had exactly the same pattern as the $\delta^{15}N$ values, reflecting similar effects of diet switching on carbon and nitrogen isotope ratios. Fig. 8.7a, b shows

a)



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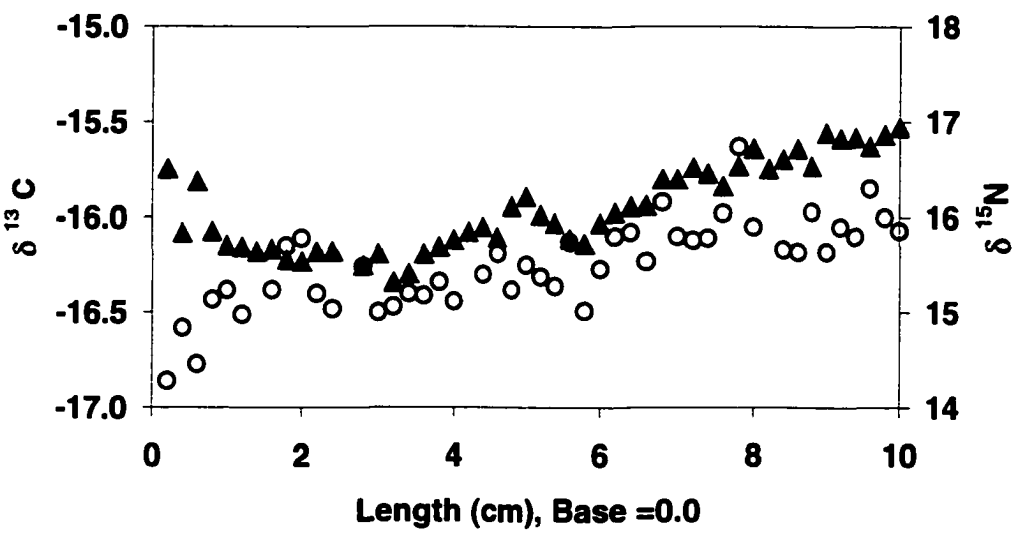
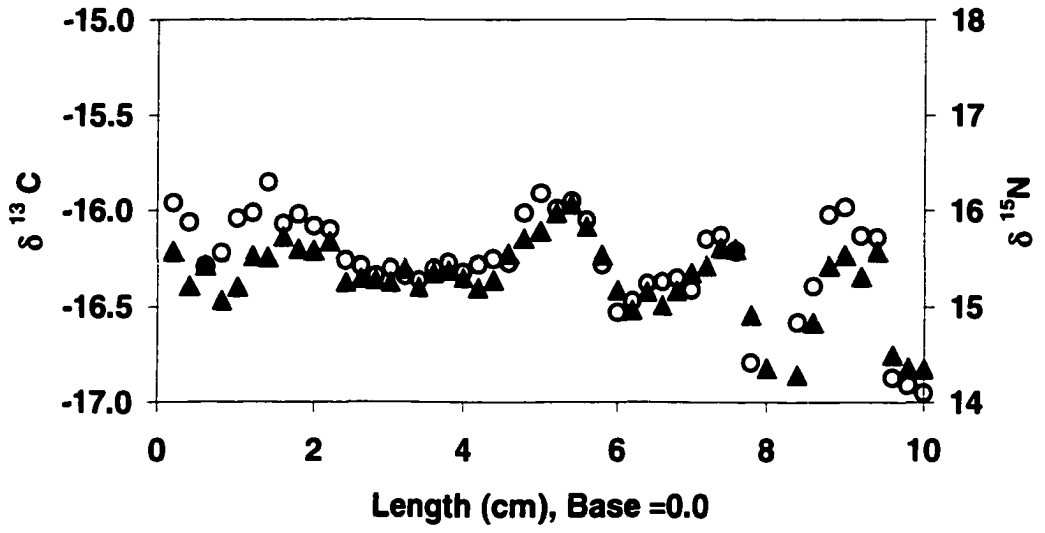


Fig. 8.5 Carbon (○) and nitrogen (▲) isotope ratios in whiskers sampled on 15 Sept. 1999. a) Snapper and b) Pender.

a)



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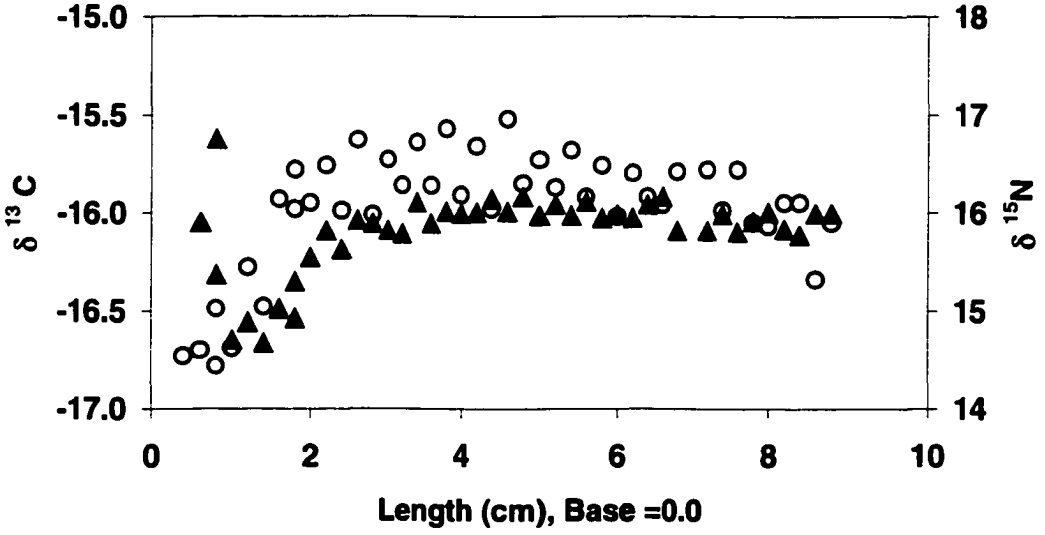
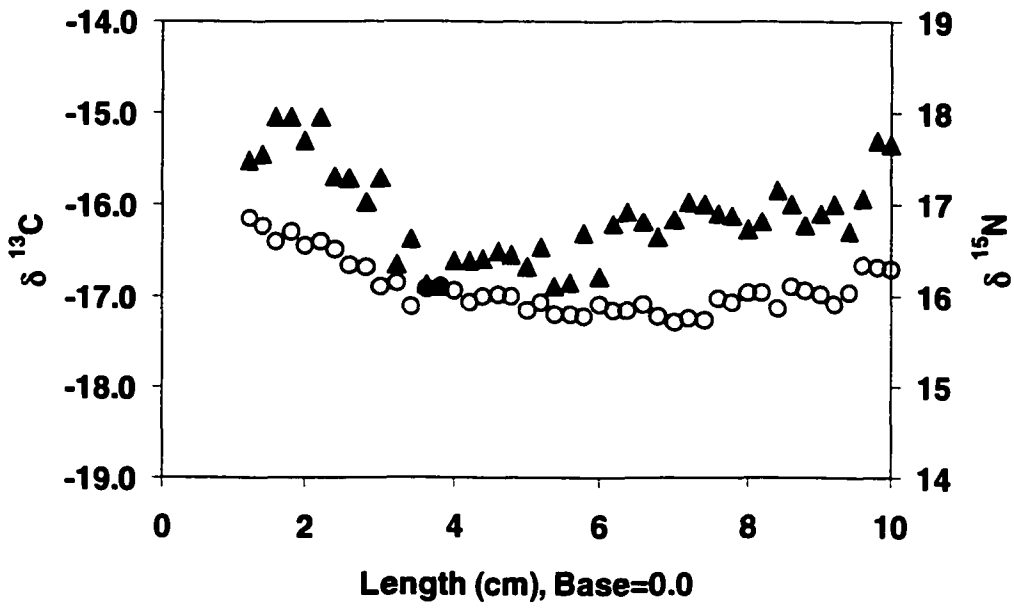


Fig. 8.6 Natural abundance variations of carbon (○) and nitrogen (▲) isotope ratios in a) a whisker from Snapper sampled on 8 Dec. 1998 and b) a whisker from Pender sampled on 19 Dec. 1998 , 4 days after ^{15}N-labeled glycine infusion. The highly enriched δ^{15}N value at the base is not shown.

a)



b)

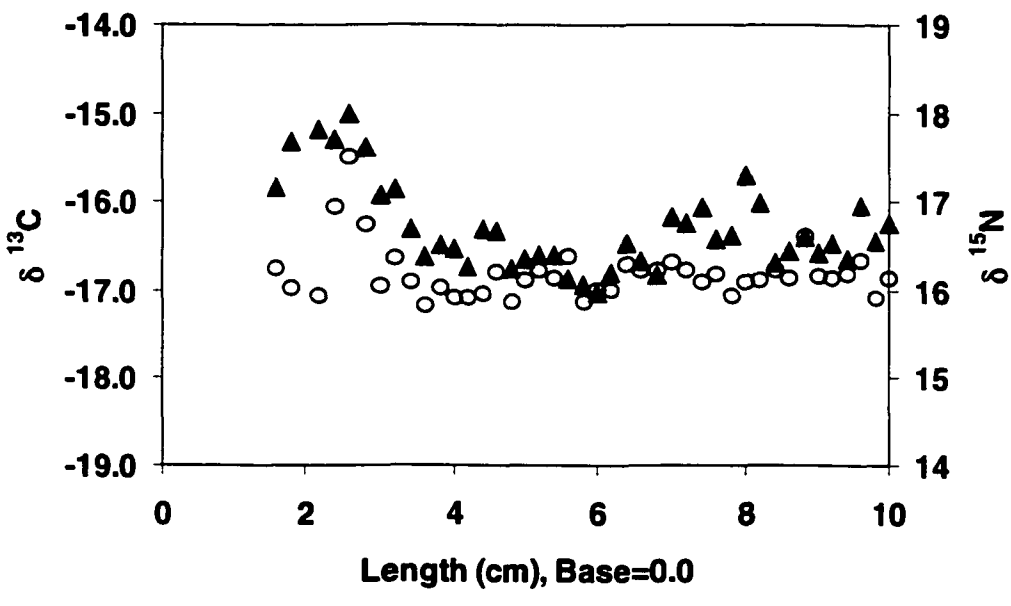


Fig. 8.7 Carbon (○) and nitrogen (▲) isotope ratios in whiskers from harbor seal Travis, sampled in Dec. 2000. a) whisker 1 and b) whisker 2.

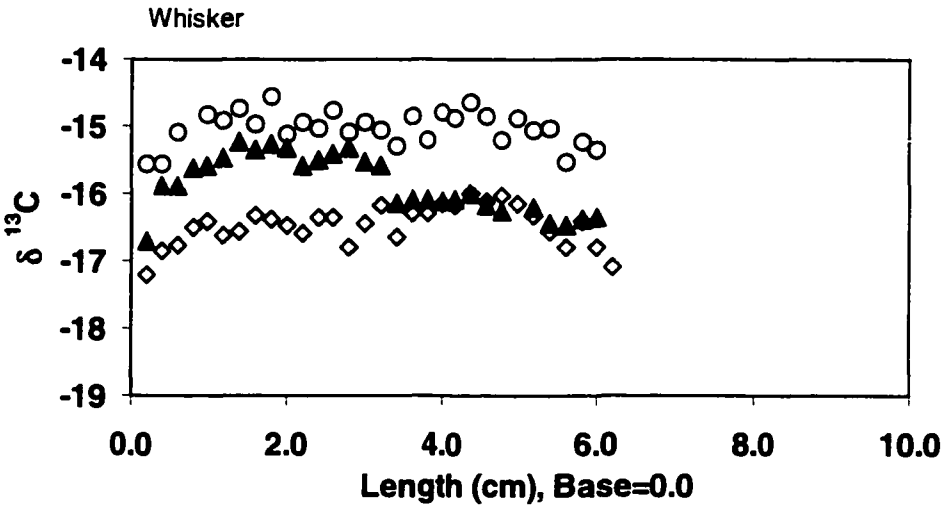
the $\delta^{13}C$ and $\delta^{15}N$ variations in two whiskers from Travis, sampled in Dec. 2000 when he accidentally died. However, the isotope ratios near the bases of whiskers are missing, since the whiskers were not clipped close to the muzzle, as determined by microscopic examination.

Fig. 8.8a shows carbon isotope ratios in whiskers sampled on 5 Sept. 2000. $\delta^{13}C$ values are depleted near the base in all three whiskers, following the same pattern as whiskers sampled on 15 Sept. 1999. The minor variations along the length of each whisker reflected the constant diet during the period of their growth and, again, supported our assumption that these whiskers were newly grown. The $\delta^{13}C$ values differed significantly among three whiskers (ANOVA, $P < 0.0001$) in response to the different fish diets. Consistent patterns of carbon isotope variations were found among whiskers, serum and red blood cells over the time period of whisker growth (Fig. 8.8b). As Fig. 8.8b shows, the highest $\delta^{13}C$ values were observed in Poco, the lowest in Pender and intermediate values in Snapper, which had a mixed diet. In addition, co-variations of $\delta^{13}C$ and $\delta^{15}N$ values were very pronounced in the Poco whisker sampled on 18 May 1999 (Fig. 8.3b). The co-variations of $\delta^{13}C$ and $\delta^{15}N$ along the length of this whisker exactly recorded the diet switch, whisker growth pattern, and ^{15}N-labeled tracer event.

DISCUSSION

Stable isotope data from both ^{15}N-labeled amino acid tracer experiments and the controlled feeding trial lead us to conclude that harbor seal vibrissae grow at highly

a)



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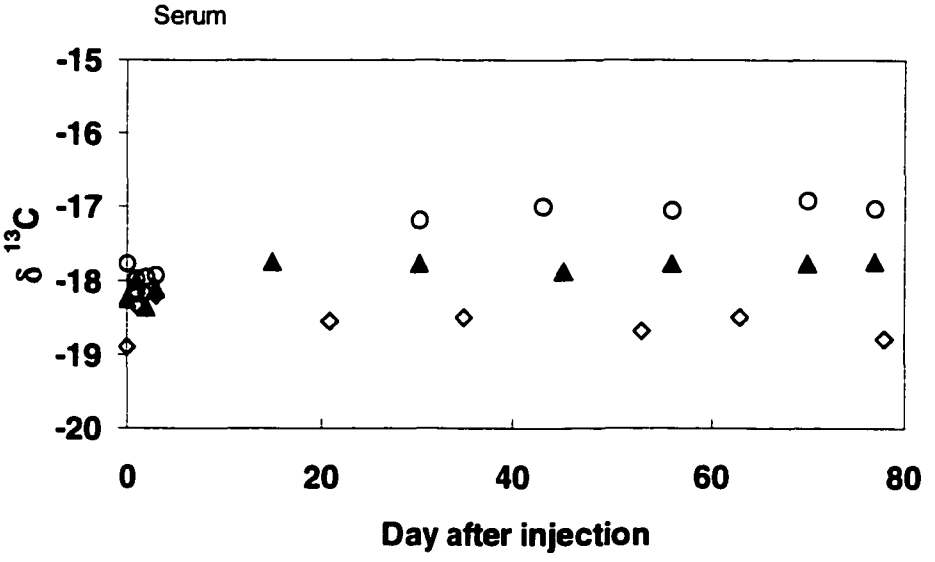

Fig. 8.8 Natural abundance variations of carbon isotope ratios in a) whiskers from the three captive harbor seals sampled on 5 Sept. 00 and b) serum during the period of whisker growth (16 May 00-5 Sept. 00). Pender (◇) on a pollock diet, Snapper (▲) on a mixed 1:1 diet and Poco (○) on a herring diet.

variable rates during different seasons. The growth rates of harbor seal vibrissae ranged from 0.075 mm/day in winter through early spring up to 0.78 mm/day in May and June for a new replacement vibrissa. It seems that harbor seals replace their vibrissae during their annual molting season. Replacement may occur in May for some seals or in late June for others. The new vibrissae can grow very quickly, reaching greater than 6 cm by September, but then grow slowly during winter and spring. An average growth rate of 0.075 mm/day was obtained from one vibrissa for the period from December to the following May.

Hirons et al. (2001) concluded that harbor seal vibrissae grew irregularly and shed annually, but they reported that the new vibrissae grew from fall to spring and that growth ceased in June. This conclusion arose from their choosing a sensitive time (4 June 1996) for ^{15}N-labeled glycine infusion. Harbor seals could have shed their vibrissae either before or after this infusion date, based on our data and inferences. Their data are, however, consistent with our estimated dates for whisker re-growth. For example, they observed that "there were no enriched glycine markers in harbor seal HS-P whiskers clipped either on 5 Nov. 1996 or 9 July 1998, in spite of high enrichments of carbon and nitrogen incorporation found in the blood". This can be explained based on the inferences that harbor seals shed their whiskers annually (for the whisker collected on 9 July 1998) and lost the marker by shedding their vibrissae after 4 June 1996 (for the whisker collected on 5 Nov. 1996). However, "one identical enriched glycine marker was observed in harbor seal HS-N whiskers sampled on 29 August 1996 and 5 November

1996, despite two dosages of enriched glycine that were infused on 9 Jan. 1996 and 4 June 1996, respectively". Hirons et al. (2001) assumed that the single marker was the result of January's infusion, based on blood isotope data. Based on the similar locations of the marker in whiskers sampled in August and November, they concluded that "the second dose given in June did not incorporate into vibrissae and vibrissae growth ceased altogether in June". However, based on our data, the marker should be still have been evident at the base of the whisker, even though vibrissa growth had ceased. A possible explanation is that the harbor seal, named HS-N, shed most of his whiskers before 4 June 1996. In that case, vibrissae sampled in August and November had both grown after 4 June, and the identical markers in these two whiskers represented June's infusion and an approximately 0.60 mm/day growth rate through August (refer to Fig. 1 in Hirons et al. 2001). This growth rate was comparable to the rapid growth rate of 0.58 mm/day found for Pender in our study. Moreover, an average growth rate of 0.08mm/day from September to April, estimated from a recaptured wild harbor seal (refer to Fig. 4 in Hirons et al. 2001), is in good agreement to a growth rate of 0.075 mm/day from December to the following May, estimated from our captive harbor seal. This suggests that there are no major differences in vibrissae growth rates between captive and wild harbor seals.

Variations of $\delta^{13}C$ and $\delta^{15}N$ values in consumer tissues can be a useful dietary indicator to track the foraging behaviors (DeNiro and Epstein 1978; 1981; Schell et al. 1989; Michener and Schell 1994; Best and Schell 1996). Co-variations of $\delta^{13}C$ and $\delta^{15}N$

values in harbor seal vibrissae reflect diets during the time periods of whisker growth, which are probably mainly late spring and summer. Since sampling of vibrissae is minimally invasive, and the isotope ratios along the length of vibrissae reflect a relatively long feeding period, vibrissae might be useful for tracking the late spring and summer feeding patterns of harbor seals if collected in early fall, despite their seasonal varying growth rates and annual replacement. Vibrissae that represent a year's growth period might be collected in early May before the annual molting, but most of the length will probably represent the prior spring and summer. Records of winter feeding would likely be confined to a narrow band near the base.

Chapter 9

Summary and Recommendations for Future Study

Carbon and nitrogen isotopic compositions of individual amino acids, ranging from –33.09‰ to –8.80‰ for $\delta^{13}C$ and –7.74‰ to 27.83‰ for $\delta^{15}N$, were measured in marine organisms from several different trophic levels, including phocids from the North Pacific, North Atlantic and Antarctic Ocean. Isotope ratios of individual amino acids differed widely within an organism, reflecting differing isotopic effects on different amino acids, due to the distinct biosynthetic pathways. Data showed a relatively conservative transfer of amino acid $\delta^{13}C$ from primary producers to top predators, offering an explanation, at the molecular level, for the commonly observed small carbon trophic enrichments between consumers and their diets. The findings support the use of $\delta^{13}C$ data in tracing carbon flow in marine ecosystems. Trophic enrichments in amino acid $\delta^{15}N$ were more pronounced than those in amino acid $\delta^{13}C$, matching the observed differences in nitrogen and carbon trophic enrichments in bulk tissues. Varying nitrogen isotopic fractionation was found for different amino acids, attributable to differing isotopic effects of transamination and oxidative deamination. Isotopic compositions of a few essential amino acids, including phenylalanine, threonine, lysine and probably histidine, were isotopically more conservative than those of most non-essential and branched-chain amino acids during their transfer along marine food chains. This

conclusion was based upon both the lesser variations of natural abundance $\delta^{15}N$ values of these essential amino acids at different trophic levels, and the very low ^{15}N enrichments measured in these essential amino acids following the tracer infusions. These findings are consistent with the fact that threonine and lysine in mammalian tissues follow irreversible oxidation, with little transamination. If the intake is not much greater than the requirements for growth and maintenance, a higher portion of dietary essential amino acids will be used directly for protein synthesis. Hence, isotopic composition in these essential amino acids will be largely conserved and these amino acids can be used as conservative "biomarkers" in stable isotopic food web studies. Further work should focus on determining the natural differences in amino acid isotopic compositions of phytoplankton across different geographic locations in the world oceans. This information would be valuable in determining habitat usage and specific prey consumption by top consumers.

Results from the controlled feeding trial demonstrate that both carbon and nitrogen isotope ratios in serum proteins of captive harbor seals varied in response to the two fish diets, Pacific herring and walleye pollock. Changes in seal physiology and protein metabolic pathways, in response to the distinct intake of dietary proteins between herring and pollock diets, may be responsible for the varying nitrogen trophic enrichments. Since isotopic fractionation mainly occurs during transamination or oxidative deamination, elevated rates of urea production and excretion, due to the high protein intake on a pollock diet, may result in greater nitrogen isotopic fractionation in

seal serum proteins. Differing carbon isotopic fractionation was also observed with these two fish diets, but was opposite to for nitrogen, with much lower trophic enrichments on the pollock diet. This was partly due to greater lipid contents in herring compared with those in pollock. These findings indicate that the commonly used constant nitrogen trophic enrichment (3.2 ‰ or 3.4 ‰) for defining the trophic position of an organism in a natural ecosystem may not be accurate in all cases, due to metabolic or physiological effects on isotopic fractionation.

Monitoring of serum free amino acid composition in two captive harbor seals over the course of the controlled feeding trial showed that serum free amino acids varied in response to changes in fish diets. The levels of several essential amino acids, particularly branched-chain amino acids, decreased when the diet was switched from herring to pollock. In contrast, glutamine and alanine, the major carriers for transaminating or transporting ammonia between peripheral proteins and the liver, increased with diet switching from herring to pollock. These patterns reflect, in a different way, changes in protein metabolic pathways of harbor seals in response to varying protein intake.

Calculations of serum protein turnover curves using a two-pool model showed an average half-life of 6.2 days for the first pool and 47.8 days for the second pool. Although the model does not necessarily correspond exactly to homogeneous physiological pools in the harbor seal, the first half-life is probably associated with the rapid tracer dilution by exchange between body free amino acids and various body

protein pools, and irreversible loss via catabolic end products, whereas the second half-life may partly represent the total body serum turnover. RBC proteins acquire the label from the formation of new RBCs, synthesized using the isotopically enriched bone marrow nitrogen pool following tracer infusions. Gradual increase of the label during the first 20 days reflects the rate of appearance of new RBCs in the bloodstream. The later, almost linear, decline after 120 days reflects gradual replacement with newly formed RBCs since the bone marrow nitrogen pool probably contains very little of the label at day 100 after the tracer administration. This result is consistent with the average life span of a red blood cell of 120 days in humans. However, the life span of red blood cells in seals was previously unknown, so this study represents a first estimate. The dietary turnover time of serum protein of captive harbor seals, defined as the time required for that specific tissue to completely incorporate the isotopic composition of the new diet following diet switching, ranged from 30 to 60 days. This agrees well with the observed metabolic turnover rate of serum protein in other mammals. These results provide an experimental foundation for using stable isotope labeled amino acid tracers for further studies of harbor seal protein metabolism. Currently, basic information on seal protein metabolism is lacking, such as their minimal protein requirements, roles of protein metabolism for energy supply and metabolic regulation in response to changing protein intake.

Tracer experiments showed that harbor seals rapidly replaced their vibrissae during summer and that the growth rate of new vibrissae can be up to 0.78 mm/day. A

much slower growth rate of 0.075 mm/day was obtained from one vibrissa during the period from December to the following May. The $\delta^{13}C$ and $\delta^{15}N$ values in vibrissae co-varied and can reflect the diet of harbor seals, mainly during the late summer to early winter period of rapid growth. Vibrissae record the diet over a longer period than serum, and can be obtained by a noninvasive sampling protocol, making them useful for tracking the feeding patterns of wild harbor seals, particularly if sampled just prior to their annual replacement during molting.

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APPENDIX 1 Amino acid abbreviations used in this dissertation

Ala	Alanine
Gly	Glycine
Ser	Serine
Asn	Asparagine
Asp	Aspartic acid
Gln	Glutamine
Glu	Glutamic acid
Arg	Arginine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Val	Valine
Lys	Lysine
Phe	Phenylalanine
Tyr	Tyrosine
Thr	Threonine
Met	Methionine
Tau	Taurine
Orn	Ornithine
a-ABA	α-amino butyric acid

APPENDIX 2 Essential and non-essential amino acids

Essential	Nonessential
Lysine	Glycine
Tryptophan	Alanine
Histidine	Serine
Phenylalanine	Aspartate
Leucine	Glutamate
Isolencine	Proline
Threonine	Proline
Methionine	Cystine
Valine	Tyrosine
Arginine	

APPENDIX 3 HPLC mobile phase elution program

ODS-II column Time (min)	Solvent A (%) Methanol	Solvent B * (%) phosphate buffer (3%THF) (pH=6.5)
0	15	85
23	70	30
28	70	30
30	15	85
35 (column conditioning)	15	85

* 0.025M 1:1 Na_2HPO_4 and NaH_2PO_4 containing 3% w/v tetrahydrofuran (THF).

NH_2 column Time (min)	Solvent A (%) Acetonitrile/water (500:70 v/v)	Solvent B (%) 0.01M phosphate buffer (KH_2PO_4, pH = 4.3)
0	90	10
5	90	10
20	70	30
27	50	50
32	90	10
37 (column conditioning)	90	10

C18 column Time (min)	Solvent A (%) Acetonitrile/water (500:70 v/v)	Solvent B (%) 0.01M phosphate buffer (KH_2PO_4, pH = 2.5)
0	3	97
10	3	97
20	30	70
21	3	97
25 (column conditioning)	3	97